Asian Pacific Journal of Allergy and Immunology

ORIGINAL ARTICLE

Pollen food allergy syndrome in Turkey: Clinical characteristics and pollen sensitization

Seçil Kepil Özdemir,[1] Selcan Özgüçlü[1]

Abstract

Background: There is limited data regarding pollen food allergy syndrome (PFAS) in Turkey.

Objectives: To investigate the clinical characteristics and possible risk factors of PFAS in Turkey, and to evaluate if there was an association between skin test reactivity to pollens and presence of PFAS.

Methods: A total of 254 consecutive adult patients with pollen sensitivity were prospectively recruited. Patients were interviewed with a questionnaire including a list of pollen-associated foods. Patients were classified as having PFAS if they reported clear allergic symptoms compatible with PFAS. All participants underwent skin prick tests (SPT) to a panel of common aeroallergens, prick-to-prick tests with culprit fresh foods were performed in patients who gave consent.

Results: Self-reported PFAS was observed in 49 patients (19.3%). The most common culprit foods were kiwi, peach, tomato, melon and watermelon. Multiple logistic regression analysis showed that potential risk factors for PFAS were having asthma (OR=2.392, *P*=0.044) and tree pollen sensitization (OR=2.904, *P*=0.004). There were no significant differences in the SPT wheal sizes to individual pollen extracts between patients with and without PFAS with a positive SPT result for that pollen extract (*P*>0.05).

Conclusions: PFAS is frequent in pollen sensitized adults in Turkey. The most commonly implicated foods are kiwi, peach,tomato, melon and watermelon, in our geographical region. SPT wheal sizes to pollen extracts seems to be similar in patients with and without PFAS.

Keywords: Grass pollen, oral allergy syndrome, pollen allergy, pollen food allergy syndrome, skin prick tests.

From:
[1] Dışkapı Yıldırım Beyazıt Training and Research Hospital, Division of Allergy and Immunology, Ankara, Turkey.

Corresponding author:
Seçil Kepil Özdemir
Izmir Dr. Suat Seren Gogus Hastalıkları ve Cerrahisi Egitim ve Arastirma Hastanesi, Allerji ve İmmunoloji Birimi, Izmir (TURKEY)
E-mail: secilkepil@gmail.com

Introduction

Pollen food allergy syndrome (PFAS) is an immunoglobulin E (IgE) mediated disease, with sensitizing reactions occurring between IgE antibodies and cross-reactive pollen allergens. The IgE originally generated in response to pollen exposure will also bind to food proteins, and producing the symptoms of PFAS.[1] Regional differences in pollen distribution and dietary habits influence the prevalence and implicated foods in PFAS. Most studies on PFAS have focused on patients with birch pollen allergy. In northern and central Europe, apple and hazelnut are most commonly implicated foods because of birch pollen sensitization while in Japan reactions to apple and peach are more common in correlation with sensitization to alder.[2,3,4] Contrary to the common birch pollen sensitization in central and northern Europe, grass allergy is the most common cause of pollinosis and tree allergy is relatively rare compared with grass and weed sensitization in Turkey, a Mediterranean area country.[5] There is limited data regarding PFAS from southeastern Europe and Turkey. Additionally, grass pollen and food cross reactivity is not as well described as tree pollen or weed pollen related PFAS. The primary objective of this study was to determine the frequency and clinical characteristics of self-reported PFAS in pollen-sensitized adults in our geographical region which is located in central Anatolia. The secondary objectives were to investigate the possible risk factors for the development of PFAS and to evaluate if there was an association between skin test reactivity

to pollens and presence of PFAS.

Methods

Study population

Two hundred fifty-four consecutive adult patients with pollen sensitivity in skin prick tests were prospectively recruited between April – August 2016. Criteria for eligibility were as follows: (a) age ≥ 18 years; (b) positive skin prick test result with a pollen extract. The study was approved by the Local Ethics Committee of the study center and written informed consent was obtained from all subjects.

All patients were interviewed by allergists with a questionnaire including a list of 38 pollen-associated foods (almond, apple, apricot, banana, black pepper, broccoli, cabbage, caraway, carrot, cauliflower, celery, chard, cherry, chickpea, coriander, cucumber, cummin, fennel, garlic, green beans, hazelnut, kiwi, lettuce, melon, mustard, onion, orange, parsley, peach, peanut, pear, pepper, plum, potato, soybean, tomato, watermelon, and zucchini). Patients were classified as having PFAS if they reported clear allergic symptoms compatible with PFAS (symptoms of oral allergy syndrome such as itching/edema of the oral mucosa) shortly (≤10 minutes) after consumption of typical pollen-associated foods. Additionally, demographic data, history of atopic disease, clinical features of the concomitant allergic disease(s), relevant medical history were recorded. The severity of the symptoms related to the primary allergic disease was assessed with visual analogue scale.

Skin prick tests

All of the participants underwent skin prick tests (SPTs) to a standard panel of common aeroallergens including grasses (*Dactylis glomerata*, *Lolium perenne*, *Phleum pretense*, *Poa pratensis*, *Anthoxanthum odaratum*), *Artemisia vulgaris*, *Plantago*, *Parieteria officinalis*, *Betulaceae* (*Alnus glutinosa*, *Betula alba*, *Carpinus betulus*, *Corylus avellana*), *Fagaceae* (*Castanea vulgaris*, *Quercus robur*, *Fagus sylvatica*), house dust mites (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), *Blatella germenica*, *Alternaria alternata*, *Cladosporium* mix (*Cladosporium cladosporioides*, *C. herbarum*), cat dander, along with positive (histamine 10 mg/ml) and negative control (Stallergenes, France). Prick-to-prick tests with culprit fresh foods were recommended for all patients with PFAS, and these tests were performed in patients who gave consent. Skin prick tests were performed on the volar forearm, and were read after 20 minutes. A wheal reaction with a mean diameter of 3 mm greater than the negative control was considered positive.

Statistical Analysis

The statistical analysis was performed using the SPSS program version 18.0 (SPSS Inc., Chicago, IL, USA). Shapiro -Wilk test was used to evaluate the distribution of quantitative data. If the data were normally distributed Student's t-test or otherwise Mann-Whitney test was performed for comparisons for quantitative variables. Chi-square test was used to compare qualitative variables. Multiple logistic regression analysis was performed to identify significant risk factors for PFAS. We used forward stepwise selection and a full model analysis that included the variables gender, familial history of atopic disease, presence of allergic comorbidities, sensitization to grass pollens, sensitization to weed pollens, sensitization to tree pollens, polysensitization, multiple pollen sensitization, age, duration of the primary allergic disease since onset, visual analogue scale scores related to the primary allergic disease. A p-value of <0.05 was considered significant.

Results

Subject characteristics

A total of 254 patients, i.e. 154 females (60.6%) and 100 males (39.4%); median (min.-max.) age 27 (18-68) years were included in the study. The vast majority (248/254, 97.6 %) of the patients had allergic rhinitis, 30 patients (11.8 %) had asthma, 17 (6.7 %) had urticaria, 6 (2.4 %) had atopic dermatitis and 1 (0.4 %) had anaphylaxis. Sixty-six (26.0%) patients had more than one allergic disease. The mean (±SD) allergic disease duration since onset was 6.8 (±5.6) years.

The vast majority of the patients were sensitized to grass pollens (243 patients, 95.7%). Sensitization to weed pollens was detected in 126 patients (49.6 %). Sensitization to tree pollens was detected in 46 patients (18.1 %). Overall, 130 patients (51.2 %) had multiple pollen sensitization to grasses, weeds and/or trees. One hundred-fourteen patients had isolated grass pollen allergy, 8 patients had isolated weed pollen sensitization and 2 patients had isolated tree pollen sensitization. Eighty-two patients (32.3 %) had another aeroallergen sensitization in addition to pollen sensitization.

Characteristics of PFAS

Self-reported PFAS was observed in 49 patients (19.3 %). Clinical characteristics of the patients with and without a history of PFAS are shown in **Table 1**. Symptoms were restricted to oropharynx in 45 (91.8 %) patients, 4 patients (8.2 %) had concurrent systemic symptoms and 1 patient (0.5 %) had anaphylaxis. The most common culprit foods were kiwi, peach, tomato, melon and watermelon (**Table 2**). Kiwi fruit and tomato were also the most common causative foods in patients with isolated grass pollen sensitization and PFAS (10/18, 55.6%; 5/18 27.8%, respectively). The majority of the patients with PFAS (30/49 patients, 61.2 %) exhibited symptoms to more than one food. In the only patient with a history of anaphylaxis, the culprit food was celery and the patient had positivity on skin prick tests with mugwort and celery. Seasonal increase of PFAS symptoms during the pollen season was reported in 5/49 patients (10.2%). Two patients (2/49, 4.1%) reported that peeling off the culprit food caused a decrement of the symptoms.

Diagnostic prick-to-prick tests with culprit fresh foods were performed in 16/49 patients and positivity on prick-to-prick tests was detected in 10/16 patients (62.5%).

Risk factors of PFAS

There were no significant differences in age, gender, duration of the primary allergic disease since onset, visual analogue scale scores related to the primary allergic disease between the patients with and without PFAS (**Table 1**). Familial history of atopic disease was more frequent in patients

with PFAS (65.3% vs. 45.9%, *P*=0.014). Regarding allergic comorbidities, frequency of allergic rhinitis, atopic dermatitis, drug allergy, food allergy, and urticaria were similar in patients with and without PFAS (**Table 1**). However, patients

Table 1. Comparison of the characteristics of the patients with and without pollen food allergy syndrome.

Characteristic	PFAS (+)	PFAS (-)	*P* value
N (%)	49/254 (19.3%)	205/254 (80.7%)	-
Age, median (min – max), years	25 (18-68)	28 (18-62)	0.381
Gender (F/M)	35/14	119/86	0.085
Presence of familial history of atopy, n (%)	**32 (65.3%)**	**94 (45.9%)**	**0.014***
Allergic comorbidities, n (%)			
Allergic rhinitis	47 (95.9%)	201 (98.0%)	0.327
Asthma	**11 (22.4%)**	**19 (9.3%)**	**0.010***
Atopic dermatitis	2 (4.1%)	4 (2.0%)	0.327
Drug allergy	5 (10.2%)	7 (3.4%)	0.059
Food allergy	3 (6.1%)	8 (3.9%)	0.448
Urticaria	4 (8.2%)	13 (6.3%)	0.749
VAS scores related to the primary allergic disease, median (min - max)	8 (1-10)	8 (1-10)	0.284
Duration of the allergic disease since onset, median (min - max), years	6.0 (1-25)	4.5 (0.1-27)	0.057
Pollen sensitization, n (%)			
Grass pollens	48 (98.0%)	195 (95.1%)	0.696
Weed pollens	25 (51.0%)	101 (49.3%)	0.826
Tree pollens	**17 (34.7%)**	**29 (14.1%)**	**0.001***

VAS: Visual analogue scale, PFAS: Pollen food allergy syndrome, *statistically significant.

Table 2. Culprit foods triggering symptoms in patients with pollen food allergy syndrome.

Culprit food	Patients with PFAS			
	SPT* positive, n	SPT* negative, n	SPT* not performed, n	Overall, n (%)
Kiwi	0	3	16	19 (38.8%)
Peach	5	1	11	17 (34.7%)
Tomato	4	0	8	12 (24.5%)
Melon	2	0	7	9 (18.4%)
Watermelon	4	0	4	8 (16.3%)
Plum	4	0	3	7 (14.3%)
Apricot	3	0	3	6 (12.2%)
Hazelnut	2	1	1	4 (8.2%)
Cucumber	1	0	2	3 (6.1%)
Orange	0	1	2	3 (6.1%)
Cherry	3	0	0	3 (6.1%)
Banana	1	0	2	3 (6.1%)
Black pepper	0	1	1	2 (4.1%)
Sunflower seed	2	0	0	2 (4.1%)
Onion	0	1	0	1 (2.0%)
Zucchini	0	1	0	1 (2.0%)
Celery	1	0	0	1 (2.0%)
Almond	0	0	1	1 (2.0%)
Apple	0	0	1	1 (2.0%)
Caraway	0	1	0	1 (2.0%)
Dill	0	0	1	1 (2.0%)

PFAS: Pollen food allergy syndrome, SPT: Skin prick test, *Skin prick test with the culprit food.

Table 3. Results of multiple logistic regression analysis to identify factors possibly associated with the presence of pollen food allergy syndrome.

Full model				Forward stepwise		
Variable	***P* value**	**Odds ratio**	**95% CI**	***P* value**	**Odds ratio**	**95% CI**
Gender	0.087	1.898	0.910-3.958	-	-	-
Asthma	0.190	1.933	0.721-5.181	**0.044***	**2.392**	**1.024-5.590**
Drug allergy	0.250	2.197	0.574-8.409	-	-	-
Urticaria	0.318	1.948	0.526-7.214	-	-	-
Food allergy	0.583	1.538	0.331-7.159	-	-	-
Polysensitization	0.481	1.293	0.633-2.643	-	-	-
Grass pollen sensitization	0.366	3.225	0.255-40.748	-	-	-
Weed pollen sensitization	0.625	0.698	0.165-2.949	-	-	-
Tree pollen sensitization	0.060	2.710	0.958-7.667	**0.004***	**2.904**	**1.412-5.972**
Familial history of atopy	0.202	1.607	0.775-3.333	-	-	-
Multiple pollen sensitization	0.768	1.283	0.245-6.723	-	-	-
Age	0.258	0.979	0.943-1.016	-	-	-
VAS scores related to the primary allergic disease	0.999	1.000	0.824-1.214	-	-	-
Duration of the allergic disease since onset	0.278	1.037	0.971-1.106	-	-	-

VAS: Visual analogue scale, *statistically significant.

with PFAS were more frequently affected by asthma (22.4% vs. 9.3%, *P*=0.01). Additionally, sensitization rates to tree pollens were higher in patients with PFAS (34.7% vs. 14.1%, *P*=0.001). Sensitization rates to grass pollens and weed pollens were not different statistically between the two groups (**Table 1**). PFAS was diagnosed in 17/46, 37.0% of the patients sensitized to tree pollens. The rates of PFAS in patients with grass pollen sensitization and weed pollen sensitization were 48/243 (19.8%), 25/126 (19.8%), respectively. The rate of PFAS was 18/114 (15.8%) in patients with isolated grass pollen sensitization. Rates of polysensitization and multiple pollen sensitization to grasses, weeds and/or trees were also statistically similar in patients with and without PFAS (38.8% vs. 30.7%, respectively, p value=0.279 for polysensitization; 61.2% vs. 48.8%, respectively, p value=0.117 for multiple pollen sensitization). Multiple logistic regression analysis of clinical features of the patients are shown in **Table 3**. Multiple logistic regression analysis with forward stepwise selection showed that potential risk factors for PFAS were having asthma (OR=2.392, p value=0.044) and tree pollen sensitization (OR= 2.904, p value=0.004) (**Table 3**).

We investigated the association between SPT result sizes to individual pollen extracts and PFAS. While comparing SPT wheal sizes to individual pollen extracts in patients with and without PFAS, we analyzed results of the patients with a positive SPT result for that specific pollen extract. Among the 243 patients sensitized to grass pollens, the SPT wheal sizes to grass pollens were similar between patients with and without PFAS (p=0.427). Among the 31 patients sensitized to *Betulaceae*, the SPT result sizes to *Betulaceae* were similar between the two groups (p=0.847). Additionally, there were no significant differences in the SPT result sizes to other pollen extracts between patients with and without PFAS (**Table 4**).

Table 4. Comparison of the skin prick test wheal diameters to individual pollen extracts in patients with and without pollen food allergy syndrome.

Pollen SPT wheal size, median mm, (min - max)	PFAS (+)	PFAS (-)	*P* value
Grass pollen mixture (n=243)	9.25 (3.00-21.00)	9.50 (3.00-22.00)	0.427*
***Artemisia vulgaris* (n=82)**	5.00 (3.00-12.50)	5.00 (3.00-16.00)	0.180*
***Plantago* (n=63)**	5.75 (3.00-10.00)	5.00 (3.00-10.00)	0.289*
***Parieteria officinalis* (n=7)**	3.75 (3.00-4.50)	8.00 (3.00-9.50)	0.113**
***Betulaceae* (n=31)**	5.00 (3.00-8.00)	5.00 (3.00-9.00)	0.847**
***Fagaceae* (n=26)**	4.50 (3.00-7.00)	4.00 (3.00-9.00)	0.836*

SPT: Skin prick test, PFAS: Pollen food allergy syndrome, *with Mann-Whitney U test, **with Student's t-test.

Discussion

In this study, we examined 254 subjects with pollen allergy from Turkey, and reported the clinical characteristics of self-reported PFAS in 49 of the patients in this sample, and compared the characteristics of the patients with and without PFAS. In our population, in which 95.7% were sensitized to grass pollens, 49.6% to weeds and 18.1% to tree pollens, the frequency of self-reported PFAS was 19.3%. Sensitization to tree pollens was associated with the highest rates of PFAS (the frequency of PFAS was 37.0%, 19.8% and 19.8% in tree, weed and grass pollen sensitization, respectively). Additionally, the result from the multiple logistic regression analysis showed that sensitization to tree pollens and presence of asthma were risk factors for PFAS. Although familial history of atopic diseases was more common in patients with PFAS, it was not a significant risk factor when it was adjusted with other associated factors in the multiple logistic regression analysis. Osterballe et al. reported that the highest probability of a clinical reaction after intake of relevant pollen-related foods was found in adults polysensitized to birch, grass and mugwort pollen and having symptoms in the pollen season.[6] Additionally, they reported that sensitization to birch pollen was associated with a higher rate of PFAS than sensitization to weed or grass pollen,[6] similar to the results in our study. The significant association between asthma and PFAS has also been reported in previous studies.[7-9] Our data, showed no differences in age, gender, duration of the pollen-related primary allergic disease, between the patients with and without PFAS. Contrary to this result, two previous studies reported longer durations of allergic rhinitis in patients with PFAS.[8,9] However, another large study found no significant difference in the duration of birch pollen allergy in patients with and without PFAS.[10]

Because of the regional differences in pollen distribution, the prevalence and implicated foods vary between different geographical regions. Based on our data, the main causative foods in PFAS were kiwi, peach, tomato, melon and watermelon in our country. To the best of our knowledge, there is only one published study about oral allergy syndrome in Turkey, however, implicated foods have not been reported in that study.[7]

Grass pollen and food cross reactivity is not as well described as tree or weed pollen related PFAS. In this study, the rate of PFAS was 15.8% and the main causative foods were kiwi and tomato in patients with isolated grass pollen sensitization. Symptoms to cross-reacting foods were reported in 4% - 19% of the subjects sensitized to grass pollens in previous studies.[6,11] Sensitization profiles to different allergen components may be responsible for the variable frequencies of PFAS in grass pollen sensitization reported from different geographical regions. In the recent multicenter study of Mastrorilli et al, which is performed in Italy, in another Mediterranean country, kiwi fruit was also the food that is most commonly triggering PFAS.[8] Another large study performed in Italy, also reported kiwi fruit to be the most frequently reported plant-derived food to be a cause of allergic reactions.[12] Allergic reactions to kiwi have been reported in association with birch and grass pollen allergies.[12,13,14] Furthermore, it has been shown that kiwi fruit contains a large number of allergens widely cross-reacting with allergens in grass and birch pollen extracts.[13]

Because of the high number of patients included in this study, we could investigate the association between SPT wheal sizes to individual pollen extracts and PFAS, by analyzing results of the patients with a positive SPT result for the examined pollen extract. There were no significant differences

in the SPT wheal sizes to the pollen extracts between patients with and without PFAS. Our findings in this respect differ from those of Ta et al, who described significantly larger sized SPT results of all the pollens combined (birch, mugwort, ragweed, tree, weed, grass mixes).[15] This discrepancy might be related to the use of different parameters while comparing SPT result sizes. Whereas, Ta et al used combined SPT results of all the pollens, we used SPT wheal sizes to individual pollen extracts of the patients with a positive SPT result for the examined pollen extract, separately. Although not confirmed in our study, patients with sensitization to multiple pollens have been shown to be more likely affected by PFAS.[6,9,11] Therefore, the larger sized SPT results of all the pollens combined in patients with PFAS compared to patients without PFAS may reflect the increased risk of PFAS in pollen-polysensitization.

In this study, we used questionnaire based interviews performed by allergists to diagnose PFAS. A major limitation of our study is that we did not perform double-blind, placebo-controlled challenges with the culprit foods to confirm the diagnosis of PFAS. However, allergic symptoms in PFAS immediately follow food ingestion and therefore they are easily recognized by the patients. It is previously shown that a positive clinical history has a high positive predictive value,[16] and a questionnaire-based diagnosis of PFAS also yielded a high positive predictive value compared to those of a protocol combining clinical history, and food challenges.[17] Additionally, because of the lability of allergens in fresh fruits, and difficulty of ensuring oral contact with adequate concentrations of the culprit food in a masking solution cause difficulty in performing double-blind, placebo-controlled challenges in PFAS.[18] Another limitation of this study is the low rate of performing diagnostic prick-to-prick tests with the culprit foods. This limitation mainly resulted from the difficulty to access the culprit fresh foods at the clinic during the first appointment. Generally, we had to ask the patient for a second appointment to obtain the culprit fresh foods, and this reduced the compliance and the proportion of the patients tested. In conclusion, self-reported PFAS is frequent in pollen sensitized adults in Turkey, which is located in the eastern Mediterranean region, and sensitization to tree pollens is associated with the highest rates of PFAS. Additionally, a significant proportion of the patients with isolated grass pollen sensitivity reports PFAS. The most commonly implicated foods in patients with PFAS are kiwi, peach, tomato, melon and watermelon, in our geographical region. SPT wheal sizes to individual pollen extracts seem to be similar in patients with and without a history of PFAS.

Acknowledgements

The authors would like to thank nurse Gülşan Taşkıran Apaydın for her help during the skin prick tests.

Conflict of Interests

The authors have no conflict of interest to declare.

References

1. Ludman S, Jafari-Mamaghani M, Ebling R, Fox AT, Lack G, Du Toit G. Pollen food syndrome amongst children with seasonal allergic rhinitis attending allergy clinic. Pediatr Allergy Immunol. 2016;27:134-40.
2. Geroldinger-Simic M, Zelniker T, Aberer W, Ebner C, Egger C, Greiderer A, et al. Birch-pollen related food allergy: clinical aspects and the role of allergen-specific IgE and IgG4 antibodies. J Allergy Clin Immunol. 2011; 127:616-22.
3. Maeda N, Inomata N, Morita A, Kirino M, Ikezawa Z. Correlation of oral allergy syndrome due to plant-derived foods with pollen sensitization in Japan. Ann Allergy Asthma Immunol. 2010;104:205-10.
4. Brown CEB, Katelaris CH. The prevalence of the oral allergy syndrome and pollen-food syndrome in an atopic paediatric population in south-west Sydney. J Paediatr Child Health. 2014;50:795-800.
5. Buyuktiryaki B, Cavkaytar O, Sahiner UM, Yilmaz EA, Yavuz ST, Soyer O, et al. Cor a 14, Hazelnut-specific IgE, and SPT as a reliable tool in hazelnut allergy diagnosis in eastern Mediterranean children. J Allergy Clin Immunol Pract. 2016;4:265-72.
6. Osterballe M, Hansen TK, Mortz CG, Bindslev-Jensen C. The clinical relevance of sensitization to pollen-related fruits and vegetables in unselected pollen-sensitized adults. Allergy. 2005;60:218-25.
7. Caliskaner Z, Naiboglu B, Kutlu A, Kartal O, Ozturk S, Onem Y, et al. Risk factors for oral allergy syndrome in patients with seasonal allergic rhinitis. Med Oral Patol Oral Cir Bucal. 2011;16:e312-6.
8. Mastrorilli C, Tripodi S, Caffarelli C, Perna S, Di Rienzo-Businco A, Sfika I, et al. Endotypes of pollen-food syndrome in children with seasonal allergic rhinoconjunctivitis: a molecular classification. Allergy. 2016;71:1181-91.
9. Asero R, Massironi F, Velati C. Detection of prognostic factors for oral allergy syndrome in patients with birch pollen hypersensitivity. J Allergy Clin Immunol. 1996;97:611-6.
10. Asero R. Relevance of pollen-specific IgE levels to the development of Apiaceae hypersensitivity in patients with birch pollen allergy. Allergy 1997;52:560-4.
11. Ghunaim N, Grönlund H, Kronqvist M, Grönneberg R, Söderström L, Ahlstedt S, et al. Antibody profiles and self-reported symptoms to pollen -related food allergens in grass pollen-allergic patients from northern Europe. Allergy. 2005;60:185-91.
12. Dondi A, Tripodi S, Panetta V, Asero R, Di Rienzo Businco A, Bianchi A, et al. Pollen-induced allergic rhinitis in 1360 Italian children: Comorbidities and determinants of severity. Pediatr Allergy Immunol. 2013;24:742-51.
13. Pastorello EA, Pravettoni V, Ispano M, Farioli L, Ansaloni R, Rotondo F, et al. Identification of the allergenic components kiwi fruit and evaluation of their cross-reactivity with timothy and birch pollens. J Allergy Clin Immunol. 1996;98:601-10.
14. Gall H, Kalveram KJ, Fork G, Sterry W. Kiwi fruit allergy: a new birch pollen-associated food allergy. J Allergy Clin Immunol. 1994;94:70-6.
15. Ta V, Scott DR, Chin WK, Wineinger NE, Kelso JM, White AA. Differential skin test reactivity to pollens in pollen food allergy syndrome versus allergic rhinitis. Alergy Asthma Proc. 2015;36:379-85.
16. Anhoej C, Backer V, Nolte H. Diagnostic evaluation of grass- and birch -allergic patients with oral allergy syndrome. Allergy. 2001;56:548-52.
17. Skypala IJ, Calderon MA, Leeds AR, Emery P, Till SJ, Durham SR. Development and validation of a structured questionnaire for the diagnosis of oral allergy syndrome in subjects with seasonal allergic rhinitis during the UK birch pollen season. Clin Exp Allergy. 2011;41:1001-11.
18. Pastorello EA, Ortolani C, Farioli L, Pravettoni V, Ispano M, Borga A, et al. Allergenic cross-reactivity among peach, apricot, plum and cherry in patients with oral allergy syndrome: An in vivo and in vitro study. J Allergy Clin Immunol. 1994;94:699-707.

ORIGINAL ARTICLE

Asian Pacific Journal of
Allergy and Immunology

APJAI

Elevated fecal calprotectin levels are associated with severity of atopic dermatitis in children

SungChul Seo,[1] So Hyun Ahn,[2] Soohyun Ri,[2] Yoonsun Yoon,[2] Jung Hye Byeon,[2] Seung Hyun Kim,[3] Wonsuck Yoon,[3] Young Yoo[1,2,3]

Abstract

Background: Recent data suggested that imbalance in gut microbiota and gastrointestinal inflammation are associated with the childhood allergic disease. Fecal calprotectin has been used for a non-invasive marker of gut inflammation.

Objective: The aim of this study was to investigate the relationships between fecal calprotectin level and the clinical severity of atopic dermatitis (AD) in children.

Methods: We enrolled 65 subjects with AD. The concentration of calprotectin was measured in each subject's fecal sample.

Results: The geometric mean fecal calprotectin level of the total subjects was 33.1(10.1-108.9) μg/g. Among the 65 subjects, 44(67.7%) showed calprotectin levels lower than 50μg/g(Group 1), and 21(32.3%) were higher than 50μg/g(Group 2). The mean SCORAD index was significantly higher in Group 2 than Group 1(31.0±16.0 vs 22.2±15.3, p=0.046). The geometric mean serum total IgE levels was higher in Group 2 compared to Group 1(361.4[31.6-992.3]IU/mL vs 175.9[44.3-699.2]IU/mL, p=0.040). The mean blood eosinophils were significantly higher in Group 2 than in Group 1(497.7[239.8-1032.8]/μL vs 281.5[121.5-652.0]/μL, p=0.034). The incidence of exposure to environmental tobacco smoke was significantly higher in Group 2 compared to Group 1(76.2% vs 47.7%, p=0.036). Geometric mean fecal calprotectin level in severe AD was significantly higher than that of mild-to-moderate AD(66.7[13.5-330.3]μg/g vs 29.4[10.1-85.6]μg/g, p=0.044). The fecal calprotectin level significantly correlated with the SCORAD index(r=0.303, p=0.014).

Conclusions: Higher fecal calprotectin levels were observed in subjects with severe AD. Elevated fecal calprotectin level as a gastrointestinal inflammatory marker may associate with childhood AD. Measurement of fecal calprotectin might be useful for assessment of severity of childhood AD.

Keywords: Atopic dermatitis, Calprotectin, Children, Gut inflammation, Gut microbiota

From:
[1] Environmental Health Center, Korea University Anam Hospital
[2] Department of Pediatrics, College of Medicine, Korea University
[3] Allergy Immunology Center, Korea University, Seoul, Korea

Corresponding author:
1. Young Yoo
Department of Pediatrics, College of Medicine, Korea University
73 Inchon-ro, Sungbuk-gu, Seoul 02841, Korea
Email: yoolina@korea.ac.kr
2. Wonsuck Yoon
Allergy Immunology Center, Korea University
73 Inchon-ro, Sungbuk-gu, Seoul 02841, Korea
Email: biokorea@korea.ac.kr

Introduction

Atopic dermatitis (AD) is a serious global disease associated with a modern lifestyle.[1] This chronic inflammatory and pruritic skin disorder affects up to 20% of children.[2] Various immune dysregulation, environmental factors, skin barrier dysfunction, and gut microbiota diversity have been emphasized as crucial pathogenesis in AD.[3] Although aberrant interactions exist between gastrointestinal inflammation, immune system, and allergic diseases,[4-6] the nature of gut inflammation underlying AD remains unclear.

The hygiene hypothesis states that reduced exposure to microbes in early childhood affects the natural development of the immune system or immune tolerance, resulting in an increased susceptibility to allergic diseases.[7] The inflamed gut epithelium with impaired barrier function has been associated with various disorders, including AD.[4] Although the direct cause and pathogenesis of AD are poorly defined, the concurrent dysbiosis in the gut microbiota has been observed in association with AD.[8] Numerous studies also suggest that imbalance in the

gut microbiota and subsequent gut inflammation are associated with allergic diseases.[5,6,8]

Calprotectin is a protein abundant in neutrophils and monocytes. Elevated concentrations of calprotectin can be measured in plasma, urine, and feces when there is ongoing inflammation.[9,10] Increased calprotectin levels have been found in chronic inflammatory bowel disease or allergic diseases.[10-12] Fecal calprotectin is a potentially important clinical test as a marker of gastrointestinal tract inflammation and has been increasingly used as a non-invasive marker for the diagnosis of bowel inflammation or gastrointestinal allergic diseases.[10,11,13]

Although many studies have reported the relationship between fecal calprotectin and gastrointestinal inflammation or allergic diseases, calprotectin has rarely been studied in AD. The aims of this study were to examine fecal calprotectin levels in children with AD and to investigate its relationship with the severity of AD.

Materials and Methods

Study subjects

Sixty-five subjects with AD aged 3 to 18 years old were recruited for this study from outpatients who attended the Allergy Clinic of Korea University Anam Hospital. The diagnosis of AD was based on the criteria of Hanifin and Rajka[14] and the SCORing of Atopic Dermatitis (SCORAD) index for assessing the severity of AD was evaluated by a pediatric allergist (Yoo Y). Among the selected subjects who met the diagnostic criteria of AD, we excluded subjects aged less than 3 years to eliminate the possible effects of breast -feeding or food allergy manifested as AD. Subjects used topical steroids, topical antibiotics, or calcineurin inhibitors for treatment of AD at the time of enrollment. None of the subjects received systemic steroids, systemic antibiotics or probiotics within 1 month before stool sample collection. Subjects with a history of food allergy or other co-morbid allergic diseases such as rhinitis or asthma, or inflammatory bowel disease were excluded from the study. We defined food allergy based on the history of subject who experienced an immediate reaction following ingestion of a specific food and increased serum food-specific IgE levels. Rhinitis was defined on the basis of subject' history and rhinologic examination. Subject who had recurrent symptoms of sneezing, rhinorrhea, and nasal stuffiness or itching apart from common cold during the previous year was considered as having rhinitis. Subject who had typical symptoms suggestive of asthma, such as a history of episodic wheezing and/or dyspnea as well as reversible airflow obstruction and/or airway hyperresponsiveness assessed by methacholine bronchial challenge test was considered as having asthma. Subjects or their parents provided written informed consent for the study. The study protocol was approved by the Institutional Review Board of the Korea University Anam Hospital (No. AN16201).

Questionnaires

The subjects or their parents completed the questionnaire which included items of age, sex, family history of allergic diseases, detailed history after food ingestion, the mode of delivery, birth weight, breastmilk feeding, and exposure to environmental tobacco smoke (ETS).

Measurement of fecal calprotectin concentration

The stool samples were obtained from 65 subjects at the time of enrollment. If subject used probiotics, stool sample was collected at least 4 weeks after quit them. Stool was collected in a screw-capped plastic container and sent to the laboratory on the same day. An extraction buffer containing citrate and urea was added on the stool sample. The samples were mixed by a vortex method for 30 seconds and homogenized for 25 minutes. One milliliter of the homogenate was transferred to a tube and centrifuged for 20 minutes. Calprotectin was analyzed using a quantitative enzyme-linked immunosorbent assay according to the manufacturer's instructions (Calprest, Eurospital SpA, Trieste, Italy). Calprotectin was expressed as micrograms per gram of feces. We divided the subjects into 2 groups according to their fecal calprotectin levels. Subjects whose calprotectin levels were lower than healthy subject's cut off level (50 μg/g)[15] were classified as Group 1 and higher than 50 μg/g were designated as Group 2.

MAST-immunoblot assay

The MAST-immunoblot assay was performed using a food panel of the AdvanSure Allergy Screen kit (LG Life Sciences, Seoul, Korea), which consists of 23 types of food allergens (soybean, milk, cheese, egg white, crab, shrimp, tuna, codfish, salmon, mackerel, pork, chicken, beef, citrus mix, peach, tomato, wheat, buckwheat, rice, barley meal, garlic, onion, and peanut), 6 types of pollen allergens (birch-alder mix, oak, rye, mugwort, ragweed, and Japanese hop), and 11 types of aeroallergens (yeast, *Alternaria*, *Aspergillus*, *Cladosporium*, *Candida*, cat, dog, cockroach, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, and *Acarus siro*). The test was performed according to the manufacturer's recommendations. The results were classified into 7 levels ranging from class 0 to class 6. Reactions more than or equal to class 2 (≥ 0.7 IU/mL) were considered positive.

Statistical analysis

Data are presented as mean±SD or geometric mean (range of 1 SD) as appropriate. Serum total IgE levels, blood eosinophils, and fecal calprotectin levels were log transformed before statistical analysis. Differences between the 2 groups were tested using Student's *t* test. Correlation analyses were performed using Pearson's correlation coefficients. All statistical analyses were performed using SPSS 20 for Windows. A p value of < 0.05 was considered significant.

Results

Among the 65 subjects with AD, 44 (67.7%) showed calprotectin levels lower than 50 μg/g (Group 1) and 21 (32.3%) were higher than 50 μg/g (Group 2). There was no significant difference in age, sex, body mass index (BMI), and birth weight between the 2 groups. The prevalence of the family history of allergic diseases, the mode of delivery, or breastfed, was not different between the 2 groups, too. The incidence of exposure to ETS was significantly higher in Group 2 compared to Group 1 (76.2% vs 47.7%, p=0.036). Twenty-one (32.3%) subjects used

topical steroids, 3 (4.6%) used topical antibiotics and 2 (3.1%) used calcineurin inhibitors for AD treatment at the time of enrollment. In fact, 23 (35.4%) subjects with positive specific IgE to food allergens were included in this study. Positive sensitization rates to food allergens were not different between the 2 groups (34.1% vs 38.1%, p=0.787) (**Table 1**). The geometric means (range of 1SD) of fecal calprotectin levels were not different between the positive- and negative food specific-IgE groups (36.6 [11.7-114.4 μg/g] vs 31.5 [9.21-107.8 μg/g], p=0.625).

Table 1. Characteristics of the subjects with atopic dermatitis

Parameters	Group 1 (N=44)	Group 2 (N=21)	p-value
Age, years	9.0 ± 3.2	8.0 ± 2.6	0.182
Boys, N(%)	14(31.8)	9(42.9)	0.417
Body mass index (kg/m^2)	17.8 ± 2.9	18.5 ± 3.2	0.348
Family history of allergy, N(%)	35(79.5)	17(81.0)	0.999
Cesarean section, N(%)	11(25.0)	9(43.9)	0.212
Birth weight, kg	3.25 ± 0.47	3.18 ± 0.49	0.567
Prematurity, N(%)	2(4.5)	2(9.5)	0.589
Breast milk feeding, N(%)	30(68.2)	13(61.9)	0.780
Environmental tobacco smoke, N(%)	21(47.7)	16(76.2)	0.036
Sensitization to food allergen, N(%)	15(34.1)	8(38.1)	0.787
Sensitization to aeroallergen, N(%)	22(50.0)	14(66.7)	0.287

Data are expressed as mean ± SD

The geometric mean (range of 1 SD) fecal calprotectin level was 16.6 (10.2-27.1) μg/g in Group 1 and 142.6 (60.9-333.6) μg/g in Group 2 (p<0.001). The mean±SD SCORAD index was significantly higher in Group 2 than Group 1 (31.0±16.0 vs 22.2±15.3, p=0.046). The geometric mean (range of 1 SD) serum total IgE levels were higher in Group 2 compared to Group 1 (361.4 [131.6-992.3] IU/mL vs 175.9 [44.3-699.2] IU/mL, p=0.040). The peripheral blood eosinophils were significantly higher in Group 2 than in Group 1 (497.7 [239.8-1032.8] /μL vs 281.5 [121.5-652.0] /μL, p=0.010). There was no significant difference in the mean age at the time of AD diagnosis between Groups 1 and 2 (2.3±2.2 years vs 2.1±1.8 years) (**Table 2**).

We compared fecal calprotectin levels in AD subjects according to the severity of disease. Fifty-five (84.6%) subjects belonged to the mild-to-moderate AD (SCORAD < 40) and 10 subjects (15.4%) belonged to the severe AD (SCORAD ≥ 40). The geometric mean (range of 1 SD) fecal calprotectin levels in severe AD (66.7 [13.5-330.3] μg/g) was significantly higher than that in mild-to-moderate AD (29.4 [10.1-85.6] μg/g, p=0.044). The serum IgE levels, sensitization rates to aeroallergens, blood eosinophils were higher in subjects with severe AD (**Table 3**). We further analyzed the 5 subjects who had a high SCORAD index (≥ 40) but a low fecal calprotectin level (< 50 μg/g). The mean±SD age (12.4±5.2 years) was older and the duration of disease (11.0±5.5 years) was much longer in the 5 subjects than in the remaining subjects. Other parameters were not significantly different in these 5 subjects.

Table 2. SCORAD indices and fecal calprotectin levels in subjects with atopic dermatitis

Parameters	Group 1 (N=44)	Group 2 (N=21)	p-value
Age at diagnosis, years	2.3 ± 2.2	2.1 ± 1.8	0.635
Duration of disease, years	6.7 ± 3.9	5.9 ± 2.9	0.404
SCORAD	22.2 ± 15.3	31.0 ± 16.0	0.046
Blood eosinophils, /μL	281.5 (121.5-652.0)	497.7 (239.8-1032.8)	0.010
Serum total IgE, IU/mL	175.9 (44.3-699.2)	361.4 (131.6-992.3)	0.040
Fecal calprotectin, μg/g	16.6 (10.2-27.1)	142.6 (60.9-333.6)	<0.001
Hemoglobin, g/dL	13.1 ± 0.9	12.7 ± 0.7	0.141
ESR, mm/hr	7.3 ± 6.5	7.1 ± 4.8	0.912
C-reactive protein, mg/L	1.6 ± 5.2	1.3 ± 2.0	0.836
Vitamin D, ng/mL	34.3 ± 8.9	34.6 ± 8.3	0.914
Zn, μg/dL	75.0 ± 9.3	75.3 ± 8.2	0.897

Data are expressed as mean ± SD or geometric mean (range of 1 SD). Group 1, calprotectin <50 μg/g; Group 2, calprotectin ≥50 μg/g. Abbreviations: SCORAD, scoring of atopic dermatitis; ESR, erythrocyte sedimentation rate

Table 3. Comparison of characteristics according to the severity of atopic dermatitis

Parameters	Mild-to-moderate AD (N=55)	Severe AD (N=10)	p-value
Age, years	8.3 ± 2.7	10.4 ± 4.5	0.189
Boys, N(%)	36(65.5)	6(60)	0.733
Family history of allergy, N(%)	44(80)	8(80)	1.000
Age at onset, years	2.4 ± 2.1	1.2 ± 1.6	0.056
Duration of disease, years	5.9 ± 3.2	9.2 ± 4.6	0.008
Sensitization to food allergen, N(%)	19(34.5)	4(40)	0.733
Sensitization to aeroallergen, N(%)	27(49.1)	9(90)	0.034
SCORAD	19.6 ± 9.9	53.0 ± 11.8	<0.001
Blood eosinophils, /μL	311.1 (137.0-706.3)	550.0 (242.3-1248.9)	0.047
Serum total IgE, IU/mL	183.1 (50.9-658.5)	632.7 (239.8-1669.0)	0.005
Fecal calprotectin, μg/g	29.4 (10.1-85.6)	66.7 (13.5-330.3)	0.044

Data are expressed as mean ± SD or geometric mean (range of 1 SD). Abbreviations: SCORAD, scoring of atopic dermatitis

We also analyzed possible correlations between fecal calprotectin levels and various biologic parameters of AD. The fecal calprotectin level significantly correlated with SCORAD index (r=0.303, p=0.014, **Figure**), but showed no statistical significance with other parameters (**Table 4**).

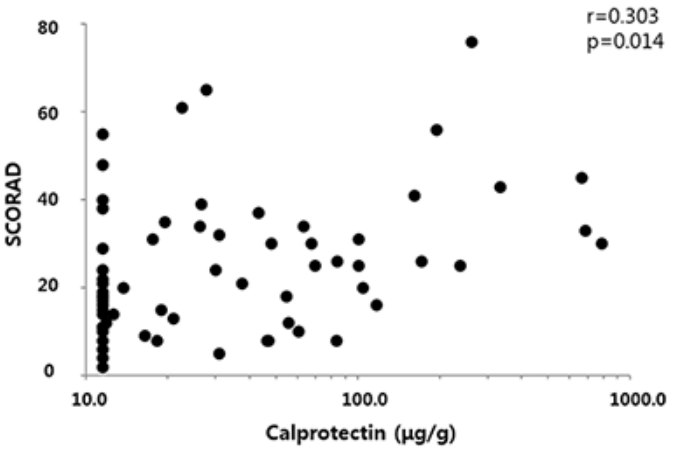


Figure. Correlation between fecal calprotectin levels and SCORAD indices in subjects with atopic dermatitis.

Table 4. Correlations between fecal calprotectin levels and other parameters

Parameters	r	p-value
Duration of atopic dermatitis, years	- 0.137	0.275
SCORAD	0.303	0.014
Blood eosinophils, /μL	0.135	0.283
Serum IgE, IU/mL	0.129	0.307
Hemoglobin, g/dL	- 0.076	0.547
ESR, mm/hr	0.017	0.891
C-reactive protein, mg/L	- 0.022	0.861
Vitamin D, ng/mL	0.058	0.644
Zn, μg/dL	0.184	0.142

Abbreviations: SCORAD, scoring of atopic dermatitis; ESR, erythrocyte sedimentation rate

Discussion

The present study demonstrated that children with high fecal calprotectin levels had more severe AD assessed by the SCORAD index. Children with elevated fecal calprotectin levels also showed higher blood eosinophils and IgE levels. Several laboratory parameters have been used to assess disease severity in children with AD but, there was no objective non-invasive severity scale until now. Thus, simple and non-invasive tests are needed as markers of disease severity in children with AD. In the present study, we showed that the measurement of fecal calprotectin might be useful for assessment of severity of AD. Fecal calprotectin has been suggested to be as a simple and reproducible marker to evaluate the inflammatory status of the gastrointestinal tract and to monitor infants with cow's milk protein allergy.[16-17]

In the present study, a reference calprotectin value of a 50 μg/g in feces is used as a cut-off level according to the study by Fagerberg *et al.*[15] Fecal calprotectin concentrations seem to be age-dependent in infancy however, healthy children aged between 4-17 years apparently exhibit constant fecal calprotectin excretions similar to those of adults.[15] AD is one of the most common clinical manifestations of food allergy and may not be completely excluded from this study. Although we did not exclude patients with food allergy by food challenge, we tried not to include subjects who experienced an immediate reaction including AD flaring after ingestion of offending food. Up to two-thirds of infants with moderate-to-severe AD show sensitization to food allergens in the first 2 years of life however, only a minor proportion of sensitized infants have concomitant IgE-mediated food allergy.[18] Thus, we excluded subjects aged less than 3 years to eliminate the possible effects of food allergy.

Increased levels of fecal calprotectin have been reported in children with inflammatory bowel disease or food allergy.[10,19,20] In patients with inflammatory bowel disease, the fecal calprotectin level closely related with mucosal gastrointestinal inflammation.[16] Fecal calprotectin levels were elevated and used to monitor mucosal healing in patients with ulcerative colitis as well.[19] Thus, fecal calprotectin has been considered a sensitive marker of mucosal healing in gut inflammation. Compared with those for inflammatory bowel or autoimmune diseases, fecal calprotectin data on allergic diseases are poorly investigated. In a recently published data,[17] fecal calprotectin levels were higher in children with non-IgE mediated cow's milk protein allergy than in controls. In addition, high fecal calprotectin levels were found in children with egg allergy.[20] The fecal calprotectin levels were almost twice higher in children with challenge-proved food allergy than in those with negative challenge results.[20] Thus, it could be useful for the diagnosis of a certain allergic diseases. However, only a few studies have addressed elevated calprotectin levels in the skin or serum in AD.[12,21] Orivuori *et al.*[22] demonstrated that infants with high fecal calprotectin levels at the age of 2 months had an increased risk of developing AD by age 6. They concluded that imbalance of the gut microbiota and intestinal inflammation in early life was associated with later development of AD.

The gut immune system is an important regulator of immune-mediated diseases, such as allergies.[4] Numerous investigators have shown that changes in the gut microbiota and intestinal inflammation have been associated with the development of allergic diseases.[6,8,13,23] The gut microbiota controls intestinal inflammation, which further modulates the development of the systemic immune system.[8,23] Altered gut microbiota induces epithelial damage resulting in increased intestinal inflammation, altered gut permeability and immunological balance, which affect the development of allergic diseases.[8,13,23] The damaged epithelium with increased permeability might allow for the passage of allergens.[24] Prolonged enhanced permeability and increased exposure to allergens might result in the subsequent development of atopic eczema.[6,24] Several studies have revealed that infants with eczema exhibit alterations in the relative levels of beneficial and potentially harmful bacteria compared to healthy infants.[5,6] Although specific molecules produced by commensal bacteria that trigger inflammatory responses in the gut are

still not well-characterized, numerous microbiota-associated molecules, including short chain fatty acid (SCFA), are known to regulate inflammatory responses through the induction of immune cells.[25] Altered gut microbiota and subsequently reduced production in SCFA can be implicated in children with AD.[8] The gut microbiota appears to play an important role in controlling not only gut specific but also distant immune responses.

It is likely that elevated fecal calprotectin plays a pathophysiological role in the inflammatory process in gut. Some researchers investigated the link between gut microbiota and fecal calprotectin as an inflammatory marker. Results of decreased fecal calprotectin after administration of probiotics for 4 weeks support that probiotics or live microorganisms may affect the host by supplying beneficial intestinal bacteria and provide health benefits.[26] Interestingly, infants with low fecal calprotectin levels shows a low *Lactobacillaceae* abundance and a high percentage of *Escherichia* in feces, which suggests early colonization of the gut microbiota regulate intestinal inflammation. This further implies that diminished gastrointestinal inflammation and subsequent improved allergic responses by administration of probiotics. In the present study, we showed higher fecal calprotectin level in subjects with severe AD. However, whether fecal calprotectin directly contributes to the pathogenetic mechanisms in children with AD remains to be explored.

Meanwhile, previous studies have demonstrated that exclusively breastfed infants have higher fecal calprotectin levels.[22,27] This could be explained by higher microbial exposure via breast milk. However, we could not find different fecal calprotectin levels in breastfed subjects. It is thought that since our subjects were 3 to 18 years old, breastfed effect did not continue until that age. In the present study, the incidence of exposure to ETS was significantly higher in subjects with high fecal calprotectin levels compared to those with low fecal calprotectin levels. Few studies have been conducted to determine the relationship between exposure to ETS and fecal calprotectin levels yet. Only 1 study demonstrated that serum calprotectin levels were higher in children with ETS exposure.[28] In that study, the researchers concluded that increased serum levels of calprotectin might be an indicator of inflammation related to ETS exposure. As children are much more prone to ETS than adults, it is believed that the effects of ETS on inflammation in children are more significant than in adults. Higher fecal calprotectin levels may not indicate the long-term exposure to ETS, but it could be at risk of long-term continuing inflammation. One thing to be clarified is that we collected data on ETS exposure at home by questionnaires instead of cotinine levels, which may have introduced some bias.

In the present study, we showed increased blood eosinophils and IgE levels in subjects with elevated calprotectin levels. However, no significant correlations were found among those 3 parameters. It is suggested that these parameters would be closely related with the clinical severity of AD but reflect different aspects of inflammatory responses in AD.

This study has some limitations. First, we chose calprotectin level of 50 μg/g as a cut-off value. Although 50 μg/g was suggested as a cut-off value in healthy children,[17] fecal calprotectin levels in children with AD were not definitely proposed. Secondly, we found no significant correlation between fecal calprotectin levels and other inflammatory markers of AD. Lastly, the diagnosis of food allergy was not made by food challenge.

The results of this study suggest that increased intestinal inflammation as measured by fecal calprotectin may be related to AD in children and useful for assessment of disease severity. Further prospectively designed randomized controlled studies are needed to confirm the role of calprotectin in monitoring the clinical severity of AD.

Acknowledgement

This study was supported in part by a grant of the Environmental Health Center for Childhood Asthma (2015-2016), Ministry of Environment, Korea, by a grant of the Basic Science Research Program through the National Research Foundation of Korea (NRF-2015R1C1A2A01055801, NRF-2015R1D1A4A01019910) and by a grant of the Korea University to Wonsuck Yoon and Young Yoo.

References

1. Worldwide variation in prevalence of symptoms of asthma allergic rhinoconjunctivitis, and atopic eczema; ISAAC. Lancet. 1998;351:1225-32.
2. Nutten S. Atopic dermatitis: global epidemiology and risk factors. Ann Nutr Metab. 2015;66 (suppl 1):8-16.
3. Flohr C, Mann J. New insights into the epidemiology of childhood atopic dermatitis. Allergy. 2014;69:3-16.
4. MacDonald TT, Monteleone G. Immunity, inflammation, and allergy in the gut. Science. 2005;25:1920-5.
5. Kosiewicz MM, Dryden GW, Chhabra A, Alard P. Relationship between gut microbiota and development of T cell associated disease. FEBS Lett. 2014;17:4195-206.
6. West CE, Ryden P, Lundin D, Engstrand L, Tulic MK, Prescott SL. Gut microbiome and innate immune response patterns in IgE-associated eczema. Clin Exp Allergy. 2015;45:1419-29.
7. Wold AE. The hygiene hypothesis revised: is the rising frequency of allergy due to changes in the intestinal flora? Allergy. 1998;53 (46 Suppl):20-5.
8. Song H, Yoo Y, Hwang J, Na YC, Kim HS. Faecalibacterium prausnitzii subspecies-level dysbiosis in the human gut microbiome underlying atopic dermatitis. J Allergy Clin Immunol. 2016;137:852-60.
9. Johne B, Fagerhol MK, Lyberg T, Prydz H, Brandzaeg P, Naess-Andersen CF, et al. Functional and clinical aspects of the myelomonocyte protein calprotectin. Mol Pathol. 1997;50:113-23.
10. Papi C, Fasci-Spurio F, Rogai F, Settesoldi A, Margagnoni G, Annese V. Mucosal healing in inflammatory bowel disease: treatment efficacy and predictive factors. Dig Liver Dis. 2013;45: 978-85.
11. Belizon CT, Paez EO, Claros AM, Sanchez IR, Gonzales AMR, Medialdea RV, et al. Faecal calprotectin as an aid to the diagnosis of non-IgE mediated cow's milk protein allergy. An Pediatr (Barc). 2016;84:318-23.
12. Jin S, Park CO, Shin JU, Noh JY, Lee YS, Lee NR, et al. DAMP molecules S100A9 and S100A8 activated by IL-17A and house-dust mites are increased in atopic dermatitis. Exp Dermatol. 2014;23:922-41.
13. Merras-Salmio L, Kolho KL, Pelkonen AD, Kuitunen M, Makela MJ, Savilahti E. Markers of gut mucosal inflammation and cow's milk specific immunoglobulins in non-IgE cow's milk allergy. Clin Transl Allergy. 2014;4:8.
14. Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. Acta Derm Venereol (Stockh). 1980;2:44-7.
15. Fagerberg UL, Loof L, Merzoug RD, Hansson LO, Finkel Y. Fecal calprotectin levels in healthy children studied with an improved assay. J Pediatr Gastroenterol Nutr. 2003;37:468-72.
16. Takashima S, Kato J, Hiraoka S, Nakarai A, Takei D, Inokuchi T, et al. Evaluation of mucosal healing in ulcerative colitis by fecal calprotectin vs. fecal immunochemical test. Am J Gastroenterol. 2015;110:873-80.
17. Beser OF, Sancak S, Erkan T, Kutlu T, Cokugras H, Cokugras FC. Can fecal calprotectin level be used as a marker of inflammation in the diagnosis and follow-up of cow's milk protein allergy? Allergy Asthma Immunol Res. 2014;6:33-8.

18. D'Auria E, Banderali G, Barberi S, Gualandri L, Pietra B, Riva E, et al. Atopic dermatitis: recent insight on pathogenesis and novel therapeutic target. Asian Pac J Allergy Immunol 2016;34:98-108.
19. Hosseini SV, Safarpour AR, Taghavi SA. Developing a novel risk-scoring system for predicting relapse in patients with ulcerative colitis: a prospective cohort study. Pak J Med Sci. 2015;31:1511-16.
20. Winberg A, Nagaeva O, Nagaev I, Lundell C, Arencibia I, Mincheva -Nilsson L, et al. Dynamics of cytokine mRNA expression and fecal biomarkers in school-children undergoing a double-blind placebo -controlled food challenge series. Cytokine. 2016;88:259-66.
21. Aochi S, Tsuji K, Sakaguchi M, Huh N, Tsuda T, Yamanishi K, et al. Markedly elevated serum levels of calcium-binding S100A8/A9 proteins in psoriatic arthritis are due to activated monocytes/macrophages. J Am Acad Dermatol. 2011;64:879-87.
22. Orivuori L, Mustonen K, de Goffau MC, Hakala S, Paasela M, Roduit C, et al. High level of fecal calprotectin at age 2 months as a marker of intestinal inflammation predicts atopic dermatitis and asthma by age 6. Clin Exp Allergy. 2015;45:928-39.
23. Noverr MC, Huffnagle GB. Does the microbiota regulate immune responses outside the gut? Trends Microbiol. 2004;12:562-8.
24. Pike MG, Heddle RJ, Boulton P, Turner MW, Atherton DJ. Increased intestinal permeability in atopic eczema. J Invest Dermatol. 1986;86:101-4.
25. Maslowski KM, Mackay CR. Diet, gut microbiota and immune responses. Nat Immunol. 2011;12:5-9.
26. Baldassarre ME, Laforgia N, Fanelli M, Laneve A, Grosso R, Lifschitz C. Lactobacillus GG improves recovery in infants with blood in the stools and presumptive allergic colitis compared with extensively hydrolyzed formula alone. J Pediatr. 2010;156:397-401.
27. Dorosko S, MacKenzie T, Connor RI. Fecal calprotectin concentrations are higher in exclusively breastfed infants compared to those who are mixed-fed. Breastfeed Med. 2008;3:117-9.
28. Cobanoglu N, Dalkan C, Galip N, Tekguc H, Uncu M, Bahceciler NN. Is calprotectin a marker of tobacco smoke related inflammation?: a pilot study in children. Inhal Toxicol. 2012;24:486-96.

ORIGINAL ARTICLE

Asian Pacific Journal of
Allergy and Immunology

APJAI

The effectiveness of newly developed written asthma action plan in improvement of asthma outcome in children

Kingthong Lakupoch, Wiparat Manuyakorn, Aroonwan Preutthipan, Harutai Kamalaporn

Abstract

Background: Providing asthma education about controller medication use and appropriate management of asthma exacerbation are the keys to improving the disease outcome. Many asthma guidelines recommend that physicians provide written asthma action plan (WAAP) to all of their asthmatic patients. However, the benefit of WAAP is unclear. Thus, we have created a new WAAP which is simplified in Thai and more user friendly.

Objective: To determine the effectiveness of the newly developed asthma action plan in management of children with asthma.

Methods: Asthmatic children who meet inclusion criteria all received the WAAP and they were followed up for 6 months with measurement of outcome variables, such as asthma exacerbation that required emergency room visit, unscheduled OPD visit, admission and school absence in order to compare with the past 6 months before receiving the WAAP.

Results: The analyzed outcomes of forty-nine children show significantly reduced emergency room visit (P-value 0.005), unscheduled OPD visit (P-value 0.046), admission days (P-value 0.026) and school absence days (P-value 0.022). Well controlled group and mild severity group were not the factors that contribute to decreased emergency room visit but step up therapy may be the co-factor to decreased ER visit.

Conclusions: The results of this study suggest that the provision of newly developed WAAP is useful for improving self- care of asthma patients and reducing asthma exacerbation.

Keywords: Asthma children, asthma exacerbation, effectiveness, emergency room visit, written asthma action plan

From:
Department of Pediatrics, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

Corresponding author:
Harutai Kamalaporn
270 Rama VI road, Thung Phaya Thai, Ratchathewi, Bangkok 10400, Thailand
Email: doctoroom@yahoo.com

Introduction

Asthma is one of the most common chronic diseases in childhood. Its prevalence has been increasing worldwide causing socioeconomic consequences. A cross-sectional study in Thai elementary school students in Bangkok reported that less than half of students with current asthma achieved control and the overall controller medication used was only 26.7%.[1] Therefore asthma exacerbation is not uncommon. Many asthma guidelines such as GINA and Thai asthma guidelines recommend physicians to provide a "written asthma action plan" (WAAP) to all children with asthma.[2] The meta-analysis in 2008 showed that information about asthma exacerbation and appropriate management can reduce the mean number of hospitalizations and emergency room (ER) visit.[3]

WAAP educates patients about warning signs and symptoms of an oncoming asthma episode, how to regularly use the peak flow meter for self-monitoring and how to realize what to do in a breathing emergency. It is controversial whether WAAP play any additional role in improvement of asthma outcomes over the routine asthma education, especially in children.[4-9]

In Thailand, WAAP for children is not popular. The survey in pediatric pulmonary and allergy staffs and fellows at Ramathibodi Hospital, Mahidol University reported that the reasons for infrequent use of WAAP included too time -consuming for doctors and difficult for parents to understand or follow the instructions. Actually, there is no specific format of WAAP worldwide. Only red, yellow, and green colors

mimicking the traffic lights are recommended. Thus, we created a new WAAP which is composed of colorful pictures of children in three different levels of comfort along with peak flow level and symptoms of asthma. In adults, 60% of asthma patients overestimate their asthma control, this may lead to incorrect management according to WAAP.[10] Therefore we use peak flow level in our action plan as an objective measurement to enhance the accuracy of asthma assessment. In addition, we inserted colorful pictures of all asthma medications and devices available in our institute adjacent to the simplified instructions concordant with standard asthma guidelines in Thai. The distinctive points of our new WAAP were its objective measurement by using peak flow level, the pictures of all medications in an easy-to-understand format, and a pre-set of instructions in Thai therefore physicians can complete the WAAP in a few minutes. Thus, we hope this WAAP would be more user friendly for both physicians and caregivers.

Our study aims to prove the effectiveness of the new WAAP in children in terms of improving self-management resulting in better asthma outcomes defined by decrement rate of ER visits, unscheduled OPD visits, hospitalization and school absence due to acute asthmatic attack.

Methods

We conducted a prospective study of children aged 5-18 years with a diagnosis of asthma who had been followed up in pediatric pulmonary and allergy subspecialty clinics in Ramathibodi Hospital from March 2015 - October 2016. The WAAP was developed and validated among pediatric chest and allergy fellows and staffs before starting to use in both clinics. For the caregivers and patient, we verbally validated the understanding of WAAP with them when recruited and every three months' time when follow up in clinics.

We recruited children who were able to perform peak flow meter and had used inhaled corticosteroid for at least 3 months prior to participation in the research study in order to ensure the correct technique of medications and devices usage. Children who developed asthma exacerbation requiring hospitalization in the past 1 month or had either cardiovascular diseases or chronic lung diseases were excluded. The informed consents were obtained from parents.

Each enrolled children received a questionnaire about baseline characteristics and history of asthma exacerbation in the previous 6 months. The newly developed WAAP was then given along with the peak flow meter.

At enrollment, the responsible physicians had to teach and confirm that the patients were able to use the peak flow meter properly. The personal best peak flow data was recorded in the WAAP and used for determining the level of severity of asthma. The physicians emphasized the patients to use controller regularly and monitor their symptoms by using peak flow twice daily as shown in WAAP. Their own symptoms were recorded. Physicians and caregivers then reviewed the WAAP together to confirm that the caregivers understand the plan for self- management for each severity level.

The outcome variables were then collected from the medical record and caregivers' interviews at the3rd and 6th month of pulmonary/allergy clinic visits respectively.

Primary outcome was the number of ER visits. Secondary outcomes were number of unscheduled OPD visits, number of admission days due to acute asthmatic attack and the number of school absence days. The definition of unscheduled OPD visits was the children with mild asthma exacerbation who chose to visit the pediatricians at outpatient department

without any appointment during working hours rather than went to ER to receive asthma management such as nebulized bronchodilator, and/or asthma medical adjustment. The definition of school absence was the children who had symptoms of asthma exacerbation and could not go to school. When 6 months follow up was completed, we evaluated the patient's WAAP adherence with a questionnaire asking about using WAAP and patients/caregivers' satisfaction.

In addition, we identified the possible factors that might confound the improvement of asthma outcome such as level of controlled, severity of asthma and step up therapy. The definition of step up therapy is the increased asthma medications following the GINA guidelines such as increased inhaled corticosteroid dosage, added on montelukast and/or long acting beta2 agonist in 6 months after receiving WAAP.

This study was approved by the committee on Human rights related to research involving human subjects, Faculty of Medicine, Ramathibodi Hospital, Mahidol University.

Study design was a prospective cohort. Categorical variables were reported as counts and compared using McNemar and Fisher exact test, as appropriate. Total ranges for some variables were provided as well. Continuous variables were analyzed by using Wilcoxon sign rank test. Data was analyzed by SPSS software version 17. A p-value of <0.05 was considered to be statistically significant.

Results

Fifty two children with asthma from pediatric pulmonary and allergy clinics at Ramathibodi Hospital were enrolled. Two of them were lost follow up, and one child was treated with immunotherapy which was a co-intervention. Of the 52 children, 49 (94%) completed the study and were included in the analysis.

Most of the children were male and two-thirds of them were 5-11 years old. Mean duration of having been diagnosed with asthma was 5.5 years prior to participating in this study. Most of the children (38 of 52; 78%) had moderate to severe persistent asthma as defined by dosage and type of inhaled corticosteroids used. However, most of them were well- controlled as defined by no asthma symptoms in the previous 4 weeks before enrollment. Allergic rhinitis was the most common comorbid and 37% of children had positive skin prick test as shown in **Table 1**.

Primary outcome

Emergency room visit

The total number of ER visit before receiving WAAP was 18 visits. After receiving WAAP, it was reduced to 3 visits. This shows a significant decrease in number of ER visits (p-value= 0.005).

The number of children who had ER visits was also significantly decreased after using WAAP. In the previous 6 months,

reporting 13 children had a total of 18 ER visits, and after using WAAP, only 3 children had a total of 3 ER visits (p-value=0.006) (**Table 2**).

Sixteen of forty-nine children (33%) had symptoms/peak flow level in yellow zone. Thirteen of sixteen children (81%) used reliever and followed the suggestions in yellow zone. All of them got better after self-management and did not have to visit the emergency room(ER). All three exacerbated children (3 of 16; 19%) did not follow the instruction in yellow zone had to go to ER.

Table 1. Baseline characteristics of the patients at enrolment

Baseline Characteristics	N=49
Gender	
male	36 (73%)
female	13 (27%)
Age	
5-11 year-old	32 (65%)
12-18 year-old	17 (35%)
Mean weight (kg)	39 (19)
Mean Height (cm)	140 (16)
Mean Duration of asthma (years)	5.5 (3.6)
Control of asthma	
Controlled	38 (78%)
Partly controlled	9 (18%)
Uncontrolled	2 (4%)
Severity of asthma	
Mild persistent	11 (22%)
Moderate persistent	23 (47%)
Severe persistent	15 (31%)
Underlying disease	
Allergic rhinitis	38 (77%)
Chronic sinusitis	1 (2%)
Food allergy	1 (2%)
GERD	4 (8%)
OSA	2 (4%)
Obesity	4 (8%)
SPT positive	18 (37%)

Table 2. Comparison of outcome variables before and after receiving WAAP

	Before (N=49)	After (N=49)	*p*-value
Total number of ER visits	18	3	N/A
Number of patients had ER visits	13 (26%)	3 (6%)	0.006*
Total number of OPD visits	4	0	N/A
Number of patients had OPD visits	4 (8%)	0	N/A
Total number of admission days	17	0	N/A
Number of patients had admission	6 (12%)	0	N/A
Total number of school absence days	55	12	N/A
Number of patients had school absence	10 (20%)	5 (10%)	0.227*

P-value calculated from McNemar test*

Secondary outcomes

Unscheduled OPD visit, Hospitalizations and School Absence

There was a significant decrease in the number of unscheduled OPD visit (p-value=0.046), the number of days of admission (p-value=0.026) and also a significant decrease in the number of days of school absence (p-value=0.022).

Four children had unscheduled OPD visits before receiving WAAP, but no children had unscheduled OPD visit after receiving WAAP. Six children had hospital admissions before receiving WAAP but no children had hospital admissions after receiving WAAP (**Table 2**).

Ten children had school absence before receiving WAAP. However, only 5 children had school absence after receiving WAAP, but there was no statistical significance (p-value 0.227) (**Table 2**).

Factors contributive to decreased ER visit

The possible factors that could affect decrement of asthma exacerbation in 6 months after using WAAP are, for example, the children in well controlled, mild severity group, step up therapy group or children who enrolled in the low season of asthma exacerbation (August to January). These groups were then analyzed in their respective subgroups. There was a significant improvement in the number of ER visits in a step up therapy group (p-value 0.009). Other factors have no statistically significant difference between groups (**Table 3**).

Table 3. Possible factors affecting decreased number of patients with ER visits 6 months after using WAAP

Factor	ER visit		*p-value*
	decreased (N=11)	unchanged* (N=38)	
Level of controlled			
- Well controlled	6 (16%)	32 (84%)	0.094
- Partly/Uncontrolled	5 (45%)	6 (55%)	
Severity of asthma			
- Mild	0	11 (100%)	0.050
- Moderate/severe	11 (29%)	27 (71%)	
Medication			
- Step up	5 (63%)	3 (37%)	0.009
- No step up	6 (15%)	35 (85%)	
Season			
- August-January	5 (17%)	25 (83%)	0.298
- February-July	6 (32%)	13 (68%)	

*Unchanged: included more ER visits in 6 months after using WAAP
P-value calculated from McNemar test*

WAAP adherence and satisfaction

At the end of the study, 46 of 49 parents (94%) answered the questionnaire about regularly using WAAP and family satisfaction. Eighty-five percent of parents usually used the WAAP. Eighty-seven percent of parents had a positive feeling of having and using WAAP. Eighty-three percent of them felt more confidence in self-management of their children's asthma symptoms (**Table 4**). About the physicians, 13 pediatric pulmonary and allergy staffs and fellows who used this WAAP reported that new WAAP had easy format to use (Score 5/5: 62%, 4/5: 38%) and most of them (69%) could complete this WAAP within 5-10 minutes.

Table 4. Parent and family evaluation after using WAAP.

	Level				
	5	**4**	**3**	**2**	**1**
Regularity of using WAAP (N=46)	29 (63%)	10 (22%)	6 (13%)	0	1 (2%)
Regularity of using ICS (N=25¥)	7 (15%)		13 (74%)	5 (11%)	0
Confidence of asthma self- management (N=46)	38 (83%)	6 (13%)	2 (4%)	0	0
Understanding of asthma severity and proper management (N=45)	29 (61%)	16 (39%)	0	0	0
Satisfaction of using WAAP (N=46)	40 (87%)	5 (11%)	1 (2%)	0	0

Note Level 5 : degree of maximum, level 1: degree of minimum
¥: the patients who already regular using ICS did not answer this question

Discussion

The majority of our population were school-aged children with moderate to severe asthma, though most of them were well controlled. At 6 months after using our newly developed WAAP, the number of children who had ER visits was significantly decreased. The result is partly consistent with the previous studies.[5,7]

GINA 2015-2017 guidelines recommend written asthma action plan to all patients with asthma to guide their self -management efforts. Self- monitoring with peak flow meter is recommended in "moderate to severe asthma" patients who need prescribed daily medication for symptom control. WAAP reminds daily use of controllers, information on how to recognize and handle worsening asthma, and when to seek for an emergency health care.

Daily peak expiratory flow monitoring can be used to assess the severity of asthma, response to medication, and detect early stages of asthma exacerbations. Patients become actively involved in managing their asthma. This may result in improved health outcomes and better asthma quality of life, especially in those who have a history of severe exacerbations or patients who poorly perceive airflow obstruction and worsening asthma.[11]

In 2009, Tatiana Espinoza-Palma et al studied 77 children who got admitted due to acute asthmatic attack.[5] This randomized controlled trial showed that asthma exacerbations, emergency visits hospitalizations significantly decreased after using asthma action plan but there was no significant difference from controlled group.

In 2013, Su Sien Wong et al studied 80 children aged 6-17 years old, most of whom were partly/uncontrolled asthma.[7] This randomized controlled trial showed significant improvement of asthma controlled test score before and after receiving action plan. However, there was no significant difference from controlled group.

Although providing written asthma action plan is recommended in many asthma guidelines, previous studies in the last decade showed that adding a WAAP can improve asthma outcome but no better than standard treatment and education.[5-8] Only one study in 2005 by Agrawal et al showed that adding WAAP can improve asthma outcome when compared with controlled group.[4] Noticeably both Agrawal et al and our study, all of the children applied the peak flow-based asthma action plan but other studies partially applied symptom-based asthma action plan. As a result, peak flow meter may be an important factor that should be tested in further studies.

WAAP also serve as a linking tool of coordination across multiple caregivers such as school nurses, and general practitioners in different clinics/hospitals. Using easy and instructive WAAP, children with asthma could receive appropriate management, especially while having acute asthmatic attack. However, symptom perception of airway obstruction is generally poor for asthmatic children and their parents. A peak flow meter is a simple device that can detect airway obstruction since early stages of acute asthma episodes. A drop in peak expiratory flow is an objective measurements of airway obstruction which can alert the patients and caregivers in starting to manage acute asthmatic attack.[12-14] In many countries, peak flow meters are provided along with WAAP for asthmatic patients without any expense. Unfortunately, they are not covered by the Thai National Health Insurance. A pilot study in 2011 revealed that the annual direct medical cost of exacerbation group is as high as twice of non-exacerbation group (14,232 Baht v.s. 7,416 Baht, p=0.02).[15] Our study demonstrated that using peak flow meter along with WAAP was feasible, useful and practical in children. The cost-effectiveness study should be done to explore whether using peak flow meter with WAAP is beneficial in decrement of asthma exacerbations. If this is the case, it should be considered to get the peak flow meters covered by Thai National Health Insurance in the future.

Although there was a systematic review in 2008 that included 4 studies between 1990-2004 that showed that symptom-based asthma action plan could reduce acute care visit more than peak flow-based plan.[16]

In addition, Agrawal et al and our study had been done in developing countries which have limited number of physicians and nurse practitioners.[3] Our finding is similar to a study in 2012 when peak flow meter was used along with WAAP with favorable outcome in quality of life. Peak flow meter may be an important factor of this distinguish outcome in children. It may increase children's awareness of their asthma symptoms that led to early intervention, resulting in improved symptom control.[11] Current medical practice with numerous patients may not be able to provide complete asthma education for every children and caregivers in a timely manner. Therefore WAAP with complete information may be a helpful tool to improve asthma outcome in this setting.

Characteristics of WAAP was one of the important factors that lead to improvement of asthma outcome.[17-19] In 2008, Matthew A. Rank et al defined the characteristics of poor and good WAAP and synthesized practical suggestions for formulating an effective and efficient WAAP.[19] For example, WAAP should have only 2-4 clearly specific action points, appropriate language, etc. The distinctive points of our newly developed WAAP are using pictures of asthma symptoms along with simple format in Thai, colorful pictures of all available medications and devices which are more understandable. It is also user-friendly. The physicians can complete it within

5 minutes. Simplified WAAP may be the tool that can remind the patients to use controller, monitor themselves regularly and become more confident to use reliever when needed. These may be the contributing factors of improved asthma outcomes in our study.

Because some confounding factors such as the well -controlled of asthma, mild severity and step up therapy could affect decrement rate of ER visits, therefore subgroup analysis was done (**Table 3**). We found that the number of children with ER visit in 6 months after using WAAP had no significant difference between well-controlled V.S. partly/uncontrolled group (p-value=0.094), mild V.S. moderate to severe persistent group (p-value=0.05). But there was a significant decrease in number of children with ER visits in a step up therapy group compared with non-step up therapy group (p=0.009). Therefore decreasing of ER visit from outcome of this study may be from newly developed WAAP and/or step up therapy, but we could not conclude which one was the most effective. We realized that stepping up of asthma medications adjusted by asthma severity level can definitely improve the disease outcome if the patients really use it. We believed that patient's compliance is the key factor. Our newly developed WAAP may play a role of a friendly reminder. It has both subjective and objective measurements of self- monitoring by using symptom-based and the peak flow meter value. We hope it can enhance the patients' awareness leading to better compliance and adherence to asthma guidelines.

Limitation of studies

Our study had no controlled group. Also some factor, such as seasonal variation of enrollment period may contribute some bias. In addition, we compared the number of outcome variables from previous 6 months that could be the recall bias.

Conclusion

Our newly developed written asthma action plan was efficient in improvement of asthma outcome in children determined by significant decrement of ER visits, unscheduled OPD visit, admission days and school absence days.

Acknowledgement

The authors would like to express gratitude to the Faculty of Medicine, Ramathibodi Hospital's research grant, and we appreciated all supports and suggestions from the pulmonary and allergy staffs, fellows and nurses.

References

1. Wanlapakorn N, Sritippayawan S, Deerojanawong J. Prevalence of asthma, level of control and factors associated with asthma control in Thai elementary school students in Bangkok. Asian Pac J Allergy Immunol. 2014;32:287-92.
2. Global Initiative for Asthma. Global strategy for asthma management and prevention 2016 [Internet]. [place unknown]: Global Initiative for Asthma; c2016 [cited 2017 Feb 3]. Available from: http://ginasthma.org/2016-gina-report-global-strategy-for-asthma-magement-and-prevention/.
3. Coffman JM, Cabana MD, Halpin HA, Yelin EH. Effects of asthma education on children's use of acute care services: a meta-analysis. Pediatrics. 2008;121:575-86.
4. Agrawal SK, Singh M, Mathew JL, Malhi P. Efficacy of an individualized written home-management plan in the control of moderate persistent asthma: a randomized, controlled trial. Acta Paediatr. 2005;94:1742–6.
5. Espinoza-Palma T, Zamorano A, Arancibia F, Bustos MF, Silva MJ, Cardenas C, et al. Effectiveness of asthma education with and without a self-management plan in hospitalized children. J Asthma. 2009;46:906–10.
6. Sunshine J, Song L, Krieger J. Written action plan use in inner-city children: is it independently associated with improved asthma outcomes? Ann Allergy Asthma Immunol. 2011;107:207–13.
7. Wong SS, Nathan AM, de Bruyne J, Zaki R, Mohd Tahir SZ. Does a written asthma action plan reduce unscheduled doctor visits in children? Indian J Pediatr. 2013;80:590–5.
8. Sheares BJ, Mellins RB, Dimango E, Serebrisky D, Zhang Y, Bye MR, et al. Do patients of subspecialist physicians benefit from written asthma action plans? Am J Respir Crit Care Med. 2015;191:1374–83.
9. John M. Kelso. Do written asthma action plans improve outcomes? Pediatric Allergy Immunol Pulmonol. 2016;29:2–5.
10. Boonsawat W, Thompson PJ, Zaeoui U, Samosorn C, Faruqi R, Poonnoi P. Survey of asthma management in Thailand-the asthma insight and management study. Asian Pac J Allergy Immunol. 2015;33:14-20.
11. Burkhart PV, Rayens MK, Oakley MG. Effect of peak flow monitoring on child asthma quality of life. J Pediatr Nurs. 2012 Feb;27:18-25.
12. Kotses H, Harver A, Humphries CT. Home monitoring in asthma self -management. J Asthma. 2006;43:649–55.
13. Harver A, Humphries CT, Kotses H. Do asthma patients prefer to monitor symptoms or peak flow? J Asthma. 2009;46:940–3.
14. Yoos HL, Kitzman H, Mc Mullen A, Sidora K. Symptom perception in childhood asthma: How accurate are children and their parents? J Asthma. 2003;40:27–39.
15. Puranitee P, Kamchaisatien W, Manuyakorn W, Vilaiyuk S, Laecha O, Pattanaprateep O, et al. Direct medical cost of Thai pediatric asthma management: a pilot study. Asian Pac J Allergy Immunol. 2015;33:296-300.
16. Zemek RL, Bhogal SK, Ducharme F. Systematic review of randomized controlled trials examining written action plans in children. Arch Pediatr Adolesc Med. 2008;162:157–63.
17. Yin HS, Gupta RS. Readability, Suitability, and characteristics of asthma action plans: examination of factors that may impair understanding. Pediatrics. 2013;131;e116.
18. Raveed K, Rohan M. Effectiveness of personalized written asthma action plans in the management of children with partly controlled asthma in Trinidad: a randomized controlled trial. J Trop Ped. 2014;60:17-26.
19. Rank MA, Volcheck GW, Li JT, Patel AM, Lim KG. Formulating an effective and efficient written asthma action plan. Mayo Clin Proc. 2008; 83:1263-70.

APJAI
Asian Pacific Journal of
Allergy and Immunology

ORIGINAL ARTICLE

Expression of cysteinyl leukotriene receptor GPR17 in eosinophilic and non-eosinophilic chronic rhinosinusitis with nasal polyps

Kun Chen,[1] Zhijian Yu,[2] Jun Yang,[1] Huabin Li[1]

Abstract

Background: The cysteinyl leukotrienes (cysLTs) are proinflammatory lipid mediators that act on the type 1 cysLT receptor (CysLT1R) in the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP). GPR17, a G protein-coupled orphan receptor with homology to the cysLT receptors, has been proposed as a damage sensor during inflammation. However, the expression and correlation of GPR17 and CysLT1R in eosinophilic CRSwNP (ECRS) and non-eosinophilic CRSwNP (non-ECRS) have not been well investigated.

Objective: To evaluate the expression of GPR17 and its correlation with CysLT1R in the 2 CRSwNP subsets.

Methods: Polyp tissues were collected from CRSwNP subjects (15 ECRS and 14 non-ECRS), and uncinate processes were collected from 12 CRSsNP subjects and 13 control subjects. The mRNA and protein levels of GPR17 and CysLT1R were examined using qRT-PCR, immunohistochemistry, and western blotting. Additionally, the correlation between GPR17 and CysLT1R at the mRNA and protein levels was evaluated. All assays were performed in a blinded manner.

Results: Polyp tissues exhibited significantly increased GPR17 expression relative to uncinate process tissues from CRSsNP patients, or healthy controls ($P=0.0012$ and $P<0.0001$, respectively). Compared with the non-ECRS subset, the ECRS subset showed significantly increased GPR17 expression. Moreover, the GPR17 expression was positively correlated with CysLT1R in nasal polyps.

Conclusions: The increased expression of GPR17 in nasal polyps and the differential expression between eosinophilic and non-eosinophilic CRSwNP subsets suggest that these subsets may have distinct pathogenic mechanisms. The positive correlation between GPR17 and CysLT1R in polyp tissues might imply substantial regulatory mechanisms that must be elucidated.

Keywords: cysteinyl leukotriene; GPR17; CysLT1R; chronic rhinosinusitis; nasal polyps

From:
[1] Department of Otolaryngology-Head and Neck Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China.
[2] Department of Otolaryngology, Zhuhai People's Hospital, Zhuhai Hospital Affiliated with Jinan University, Zhuhai, China.

Corresponding author:
1. Huabin Li
Department of Otolaryngology-Head and Neck Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, No. 1665, Kongjiang Road, Shanghai, 200092.China.
E-mail: allergyli@163.com
2. Jun Yang
Department of Otolaryngology-Head and Neck Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, No. 1665, Kongjiang Road, Shanghai, 200092.China.
E-mail: yangjun@xinhuamed.com.cn

Introduction

Chronic rhinosinusitis (CRS) is a common disease that affects 4-10% of the global population, which is characterized by chronic inflammation of the mucosa of the nose and paranasal sinuses that persists for a minimum of 12 weeks. CRS can generally be classified into 2 subtypes: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP).[1,2] Recently, CRSwNP was proposed to be a heterogeneous disorder with variable clinical manifestations in different regions, and CRSsNP is considered an inflammatory condition that is associated with a Th1-immune response.[2] Based on the extent of tissue eosinophilia, it has been proposed that CRSwNP be subclassified into eosinophilic and non-eosinophilic subtypes, each of which is characterized

by distinct degrees, therapeutic strategies, and prognoses.[3,4] Despite numerous studies designed to clarify the cellular and molecular features of differential chronic inflammation between patients with eosinophilic CRSwNP (ECRS) and patients with non-eosinophilic CRSsNP (non-ECRS), the molecular mechanisms underlying the discrepancy remain largely unknown.[5]

The P2Y-like, G protein-coupled receptor GPR17 is phylogenetically located at an intermediate position between P2Y and cysteinyl leukotriene (cysLT) receptors; it is a dual receptor for uracil nucleotides and cysLTs (LTC_4, LTD_4, and LTE_4) and is highly expressed in organs undergoing ischemic damage.[6,7] Additionally, the *in vivo* inhibition of GPR17 by cysLT receptor antagonists dramatically reduces ischemic damage in a rat focal ischemia model, suggesting that GPR17 is the common molecular target mediating brain damage by cysLTs.[7] These proinflammatory lipid mediators (LTC_4, LTD_4, and LTE_4) have been specifically implicated in human bronchial asthma via the type 1 cysLT receptor (CysLT1R), and antagonism of CysLT1R and GPR17 with the specific inhibitor montelukast reduces the levels of inflammatory cytokine expression.[8,9] However, a recent study showed that GPR17 negatively regulates CysLT1R functions in allergic pulmonary inflammation.[10] We thus propose a possible pathological role of GPR17 in nasal polyps; this is the first study to examine the expression of GPR17 in sinus tissues.

Significant pathologic findings in nasal polyp tissues include intense eosinophilic inflammation, leading to a need for more aggressive courses of medical treatments and surgical therapies.[11] In recent years, there has been increasing interest in the potential role of cysLTs in the pathogenesis of CRSwNP. For instance, Steinke et al.[11] showed that cysLTs levels are significantly increased in ECRS patients but not in non-ECRS patients and healthy controls. Our previous study also revealed the differential expression of cysLTs and its receptors (CysLT1R and CysLT2R) between the two CRSwNP subsets.[13] It is thus necessary to address whether GPR17, as one of the cysLT receptors, is associated with the phenotype of CRSwNP.

Materials and methods

Patients

Adult patients with CRSwNP (15 ECRS and 14 non-ECRS patients), or CRSsNP (12 patients) were enrolled from the Department of Otolaryngology, Head and Neck Surgery of Xinhua Hospital, Shanghai Jiaotong University School of Medicine. The diagnoses of CRSwNP and CRSsNP were made based on medical history, nasal endoscopy, and computed tomography (CT) scans of the paranasal cavities, in accordance with the current European guidelines on rhinosinusitis and nasal polyps.[1] Skin-prick tests with a panel of aeroallergens (pollens, dust mites, pets, molds, etc.) were performed to evaluate the atopic status of the patients. Asthma was diagnosed by a pneumologist based on the disease history and an evaluation of airway responsiveness. None of the subjects used oral or nasal steroids or other immune-modulating drugs for 4 weeks prior to surgery. Patients who had a previous history of acute infection, fungal sinusitis, cystic fibrosis, antrochoanal polyps, or gastroesophageal reflux disease were excluded from the study. During endoscopic surgery, polyp tissues were sampled from patients with CRSwNP, whereas uncinate process tissues were sampled from patients with CRSsNP. As healthy controls, 13 patients undergoing septoplasty because of anatomic variations were enrolled, and the uncinate process was sampled during surgery. The demographic data of all subjects enrolled in this study are listed in **Table 1**. This study was approved by the ethics committee boards at the Xinhua Hospital Affiliated Shanghai Jiaotong University School of Medicine, and written informed consent was obtained from each subject. According to eosinophil levels, the CRSwNP patient population was divided into ECRS and non-ECRS.[14] In summary, the cutoff value separating ECRS from non-ECRS was set at 8 eosinophils/high-power field (HPF); a polyp with ≥8 eosinophils/HPF was defined as ECRS. All the laboratory assays were performed in a single-blinded manner.

Table 1. Subjects' characteristics

Characteristics	Control	CRSsNP	NP
Subjects (no.)	13	12	29
Sex (M/F)	8/5	9/3	19/10
Age (y)	45 (28-62)	42 (26-60)	52 (31-74)
Duration (y)	—	5 (3-9)	7 (2-12)
Asthma history, yes/no	—	1/11	3/26
AERD history	—	—	—
Smoking history, yes/no	—	3/9	8/21
Skin prick test response, positive/negative	—	2/10	9/20

CRSsNP, chronic rhinosinusitis without nasal polyps; NP, nasal polyps; AERD, Aspirin-exacerbated respiratory disease; M, male; F, female.

Each specimen was divided into 3 pieces for further analysis. The first was stored immediately in RNA-stabilizing solution (RNAlater; Tiangen, Beijing, China) for subsequent RNA extraction; the second was fixed with 4% paraformaldehyde and then embedded in paraffin wax for immunohistochemical (IHC) staining; and the third was stored immediately at -80°C for western blot analysis. In addition, the correlation between GPR17 and CysLR1R levels in polyp tissues was evaluated.

qRT-PCR

The mRNA expression levels of GPR17 and CysLT1R were evaluated using quantitative real-time polymerase chain reaction (qRT-PCR) analysis as previously described.[14] Briefly, total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Reverse transcription was performed, in which cDNA for quantitative PCR was synthesized from 2 μg of total RNA using an oligo (dT) 18 primer and M-MLV reverse transcriptase (Takara, Dalian, China). RNA integrity and the success of the reverse transcription reaction were monitored by PCR amplification of β-actin transcripts. Messenger RNA (mRNA) expression was determined using the ABI PRISM 7500 Detection

System (Applied Biosystems, Foster City, CA) with SYBR Premix Taq (Takara, Dalian, China). The primer sequences were as follows: GPR17 forward, 5'-CCC TGG CTC TGT GGC TTT TC-3'; GPR17 reverse, 5'-TCT CGT TGG TTT TCC CTT CG-3'; CysLT1R forward, 5'-ACT TCC GCA ATC AAG TGT ATT-3'; CysLT1R reverse, 5'-ATT GCC AAA GAA GCC TAC AAC-3'; β-actin forward, 5'-TGT GTT GGC GTA CAG GTC TTT G-3'; β-actin reverse, 5'-GGG AAA TCG TGC GTG ACA TTA AG-3'. The qRT-PCR amplification protocol consisted of 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension cycles at 60°C for 45 seconds each. Melting curve analysis was used to control for amplification specificity. The mean cycle threshold (Ct) values were normalized to those of β-actin, and the relative mRNA levels of the target genes were analyzed using the $2^{-\triangle\triangle Ct}$ method. Experiments were performed in triplicate for each data point.

IHC staining

Immunohistochemical staining was performed using a peroxidase-labeled streptavidin-biotin technique as described.[14] Briefly, paraffin-embedded human nasal tissues were cut into 4-μm sections and placed onto glass slides. The sections were incubated overnight at 4°C with primary antibodies against GPR17 and CysLT1R with a dilution of 1:200 (Abcam, Cambridge, MA). Subsequently, each of the sections was incubated with a secondary antibody and then detected with streptavidin-biotin-horseradish peroxidase complex (Zhongshan -jinqiao, Beijing, China). Immunostaining was considered positive when brown cells were obtained after treatment with 3% 3,3'-diaminobenzidine reagent. Negative controls were performed by replacing the primary antibodies with normal IgG at appropriate concentrations. The sections were examined and scored by 2 independent observers who were blind to the diagnosis and clinical data. The numbers of positive cells were counted in 5 randomly selected HPFs (×400 magnification) and averaged.

Western blot analysis

GPR17 and CysLT1R protein levels were determined by western blot analysis. Briefly, the tissues were dissected on ice and homogenized by sonication in radioimmunoprecipitation assay (RIPA) lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The protein concentration in the supernatants was determined using the BCA method. Samples containing 15 μg of protein were boiled, subjected to SDS-PAGE in 10% Tris-glycine gels, and electrophoretically transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% fat-free milk in Tris-buffered solution (TBS) containing 0.05% Tween-20 for 1 hour at room temperature before being incubated with primary antibodies against GPR17 and CysLT1R at a dilution of 1:2,000 (Abcam, Cambridge, MA) overnight at 4°C. The membrane was then washed and incubated with a horseradish peroxidase-linked secondary antibody before being processed with the enhanced chemiluminescence (ECL) reaction kit and exposed on medical film. The relative band density of the target protein relative to the β-actin protein was determined with Bio-Rad Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Hercules, Calif).

Statistical analysis

Data were expressed as medians and interquartile ranges. These data were analyzed using the Kruskal-Wallis H test and the nonparametric Mann-Whitney U test. The correlation between GPR17 and CysLT1R expression levels in the same nasal polyps was assessed using the Spearman rank correlation test. A P value of less than 0.05 was considered statistically significant.

Results

GPR17 and CysLT1R mRNA levels in nasal tissues

To determine whether cysLT receptor molecules are altered in polyp tissues from patients with CRSwNP, we first determined the expression levels of GPR17 and CysLT1R in nasal tissues. As indicated by qRT-PCR (**Figure 1A, B**), the GPR17

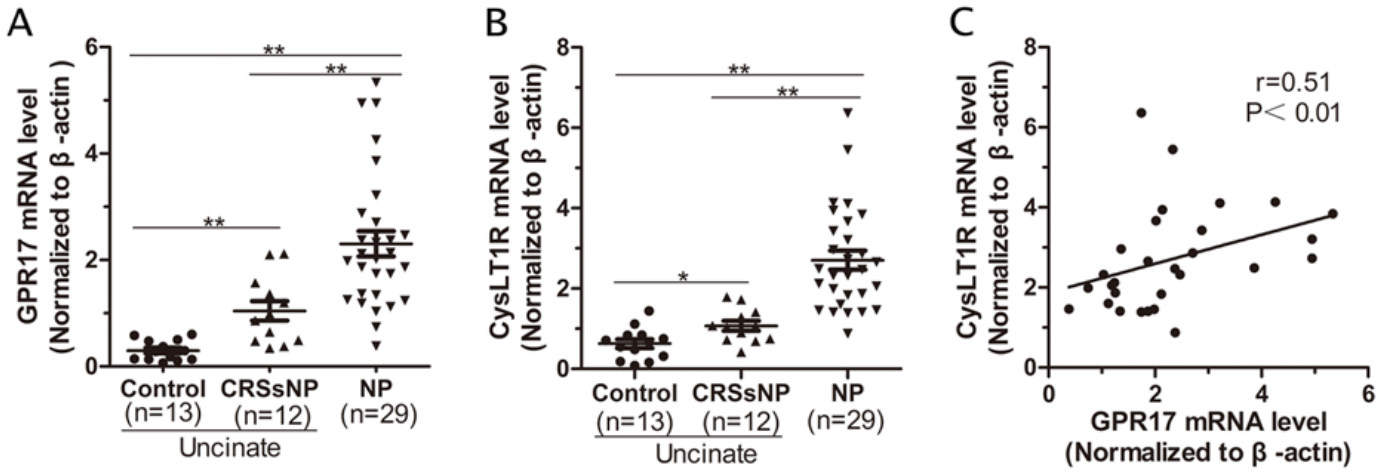


Figure 1. CysLT1R and GPR17 mRNA expression in nasal tissues. The GPR17 (A), and CysLT1R (B) mRNA levels were significantly increased in polyp tissues compared with the observed values seen in the other subgroups. (C), Correlation between the GPR17 and CysLT1R mRNA levels in the same NP tissues. The results are expressed as medians (interquartile ranges). CRSsNP, chronic rhinosinusitis without nasal polyps; NP, nasal polyp. *, P<0.05; and **, P<0.01.

and CysLT1R mRNA levels in polyp tissues from patients with CRSwNP were significantly higher than in uncinate tissues from patients with CRSsNP or from control subjects (P<0.01). Interestingly, significant differences in GPR17 and CysLT1R mRNA expression were observed between uncinate tissues from patients with CRSsNP and healthy controls (**Figure 1A, B**). Additionally, there was a positive correlation between the mRNA levels of GPR17 and CysLT1R in nasal polyps (**Figure 1C**; r=0.51, P<0.01).

GPR17 and CysLT1R immunoreactivity in nasal tissues

As indicated by IHC (**Figure 2A**), GPR17 and CysLT1R exhibited strong cytoplasmic staining that was mainly located in the inflammatory cells in the lamina propria of polyp tissues. In contrast, we found that GPR17 and CysLT1R immunoreactivity was quite weak in normal tissues. The mean number of GPR17- and CysLT1R-positive cells was significantly increased in nasal polyps from patients with CRSwNP relative to uncinate tissues from patients with CRSsNP or from control subjects (**Figure 2B, C**). Furthermore, the numbers of GPR17$^+$ cells and CysLT1R$^+$ cells in the polyp tissues were positively correlated (**Figure 2D**; r=0.44, P<0.05).

GPR17 and CysLT1R protein levels in nasal tissues

Using western blot analysis (**Figure 3A-C**), we found that the GPR17 and CysLT1R protein levels were significantly increased in polyp tissues from patients with CRSwNP relative to uncinate tissues from patients with CRSsNP or from healthy

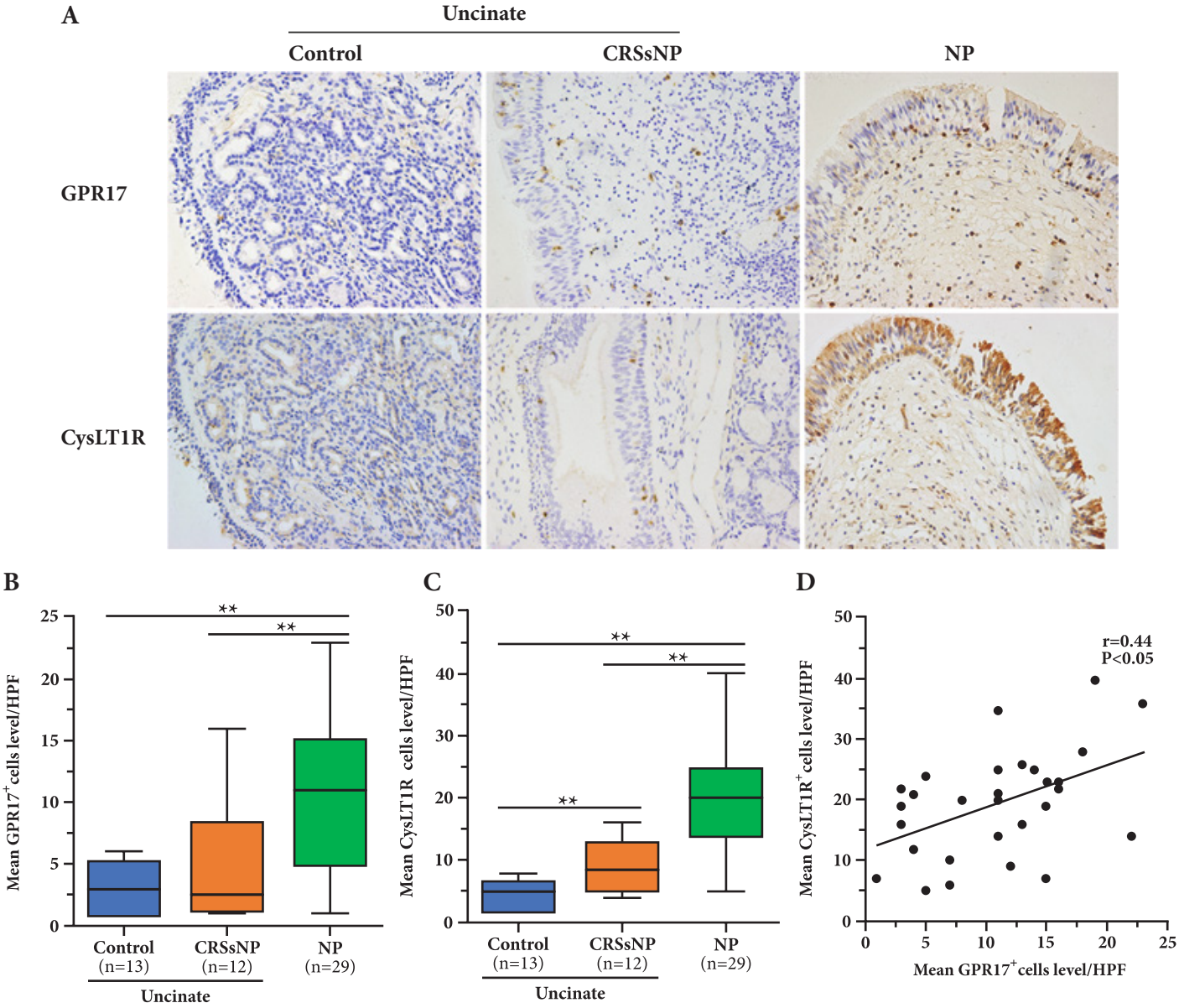


Figure 2. Immunoreactivity of CysLT1R and GPR17 in nasal tissues. (A) Representative IHC staining of GPR17 and CysLT1R in the 3 subgroups (magnification, ×200). (B, C) Quantitative analysis of GPR17$^+$ cells and CysLT1R$^+$ cells in healthy controls, CRSsNP patients, and NP patients. (D) Correlation between GPR17$^+$ and CysLT1R$^+$ cells counts in the same polyp tissues. The data are expressed as medians (interquartile ranges). IHC, immunohistochemistry; HPF, high-power field; CRSsNP, chronic rhinosinusitis without nasal polyps; NP, nasal polyp. **, P<0.01.

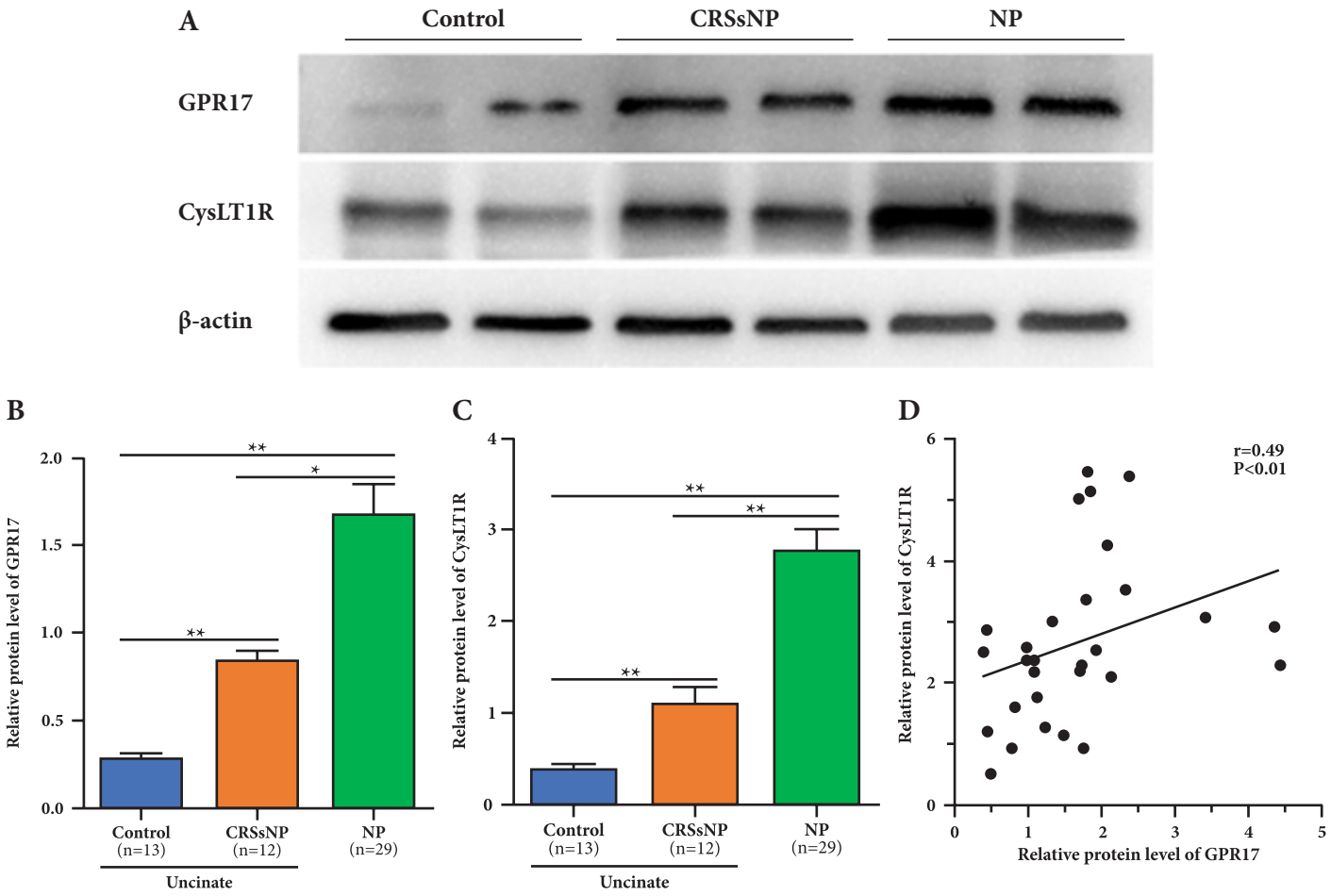


Figure 3. Protein levels of GPR17 and CysLT1R in nasal tissues. (A) Representative western blot analysis of GPR17 and CysLT1R in the 3 subgroups. (B, C) Quantitative analysis of GPR17 and CysLT1R protein levels in normal controls, CRSsNP patients, and NP patients. (D) Relative protein levels of CysLT1R and GPR17 in the same nasal polyps were positively correlated. Data are expressed as medians (interquartile ranges). CRSsNP, chronic rhinosinusitis without nasal polyps; NP, nasal polyp. *, P<0.05, and **, P<0.01.

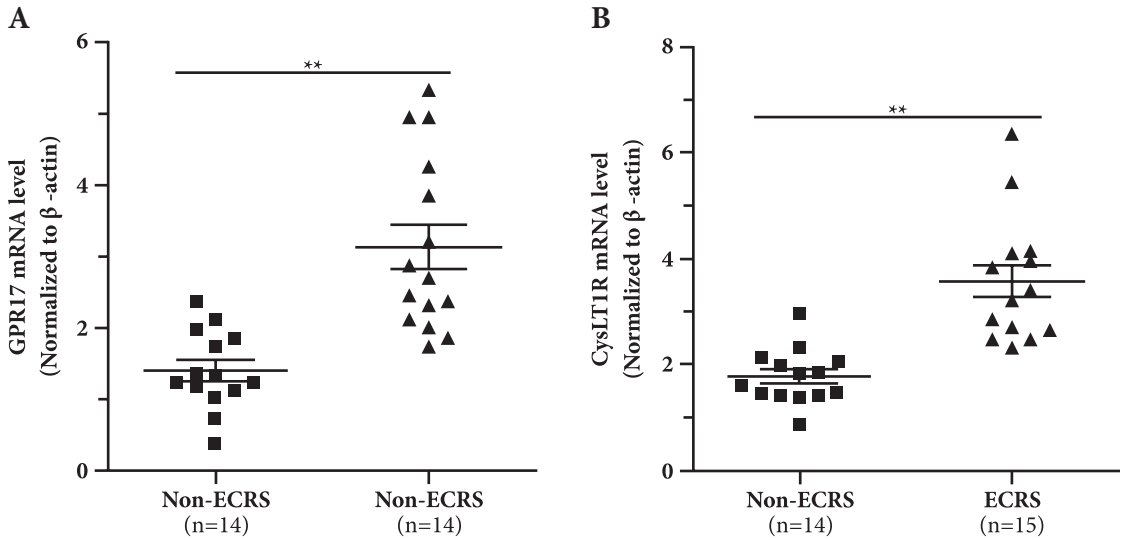


Figure 4. mRNA expression and distribution of GPR17 and CysLT1R in eosinophilic and noneosinophilic polyp tissues. (A, B) mRNA expression of CysLT1R and GPR17 in polyp tissues.

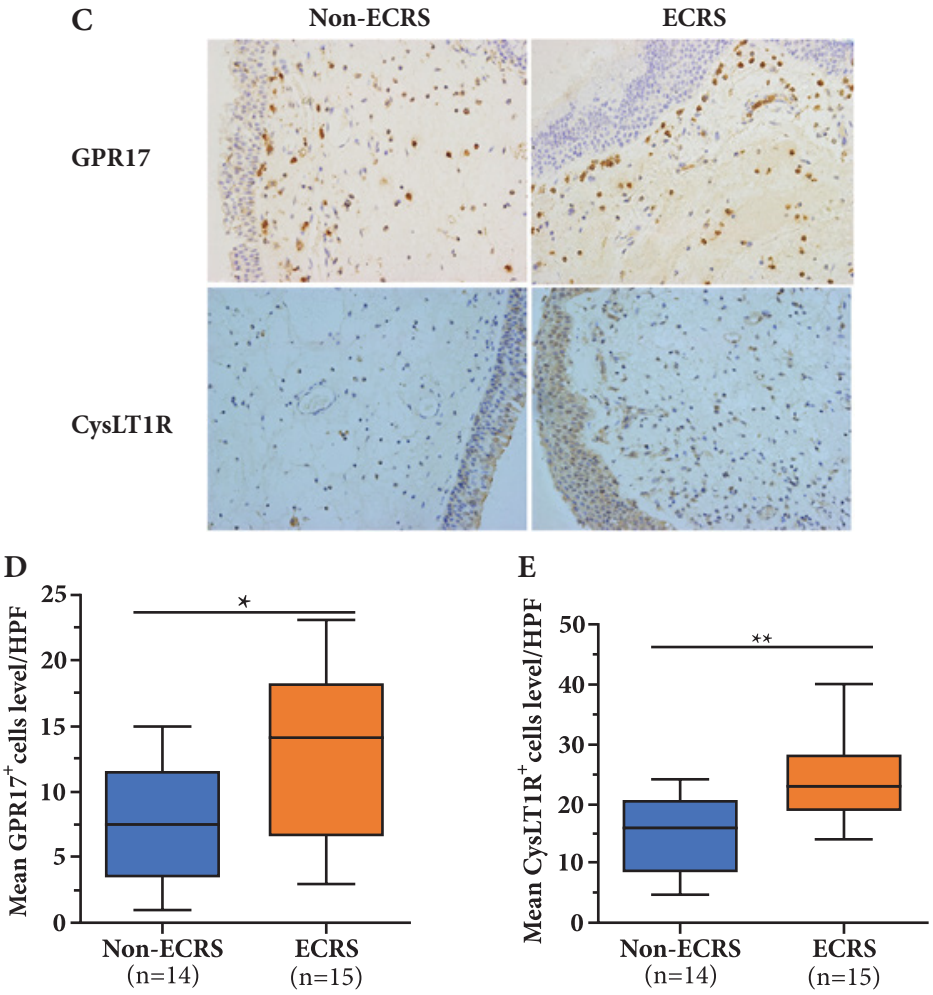


Figure 4. (Continued) (C) Representative GPR17 and CysLT1R staining (×200) in nasal tissues from patients with ECRS, patients with non-ECRS, and control subjects. (D, E) Quantitative analysis of $GPR17^+$ and $CysLT1R^+$ cells in patients with ECRS and non-ECRS. The data are expressed as medians (interquartile ranges). HPF, high-power field; ECRS, eosinophilic chronic rhinosinusitis with nasal polyp; non-ECRS, noneosinophilic chronic rhinosinusitis with nasal polyp. *, P<0.05; **, P<0.01.

controls (P<0.01). Furthermore, we found a positive correlation between protein levels of GPR17 and CysLT1R in nasal polyps (**Figure 3D**; r=0.49, P<0.01).

GPR17 and CysLT1R are differentially expressed in the eosinophilic and non-eosinophilic CRSwNP subsets

When comparing the GPR17 and CysLT1R mRNA levels between the 2 CRSwNP subsets, we found that the mRNA levels were significantly higher in the ECRS patient subset than that in the non-ECRS subset (**Figure 4A, B**; P<0.01). Furthermore, we found that the mean number of GPR17- and CysLT1R-positive cells was significantly greater in ECRS patients than in non-ECRS patients (**Figure 4C-E**; P<0.05). The GPR17 and CysLT1R protein levels were also clearly elevated in polyp tissues from patients with ECRS relative to those in non-ECRS patients (**Figure 5A-C**; P<0.01).

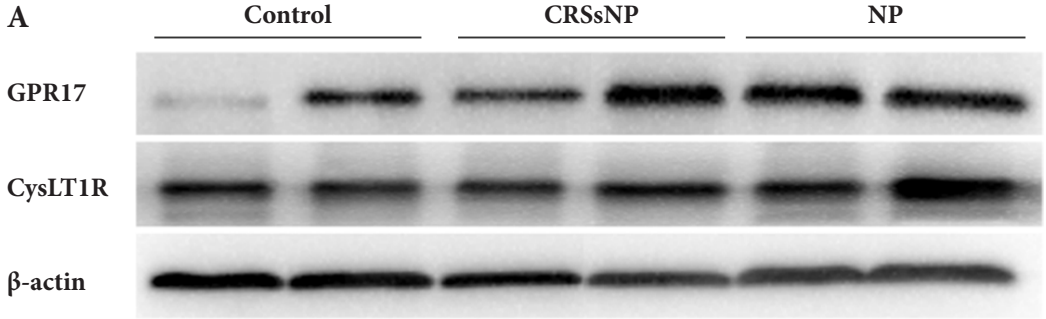


Figure 5. Protein levels of GPR17 and CysLT1R in eosinophilic and noneosinophilic polyp tissues. (A) Representative western blot analysis of GPR17 and CysLT1R in the ECRS and non-ECRS groups.

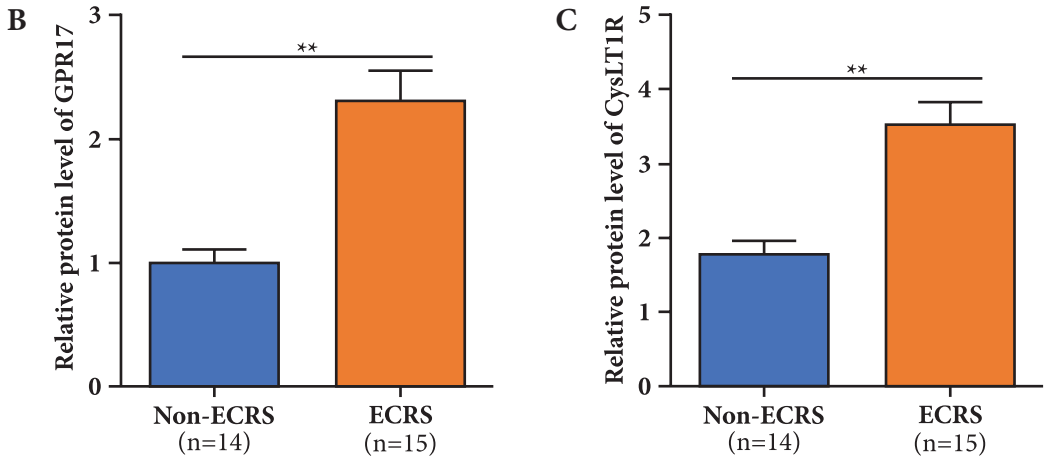


Figure 5. (Continued) (B, C) Quantitative analysis of GPR17 and CysLT1R protein levels in non-ECRS patients and ECRS patients. The data are expressed as medians (interquartile ranges). ECRS, eosinophilic chronic rhinosinusitis with nasal polyp; non-ECRS, noneosinophilic chronic rhinosinusitis with nasal polyp. **, P<0.01.

Discussion

CRSwNP is traditionally considered a chronic inflammatory disease of the upper airways, with an enhanced Th2 response, eosinophilic inflammation, and massive edema. The endotyping patients of CRSwNP patients by histopathological classification (eosinophilic vs. non-eosinophilic) rather than by simple clinical phenotypes (CRSwNP vs. CRSsNP) reflects a change in the understanding the underlying inflammatory processes in order to design more-effective approaches to disease management.[3] On the basis of such immunopathogenic findings, corticosteroids have been accepted as a central element in treatment strategies and have proven to be quite effective for CRSwNP patients. Corticosteroids show a potent anti-inflammatory function by suppressing the gene transcription of proinflammatory products and reducing airway inflammatory cell infiltration and function. Compared with a placebo, corticosteroids have improved overall symptom scores, reduced polyp sizes, decreased polyp scores, and prevented polyp recurrence after sinus surgery.[15,16] Nevertheless, even with corticosteroid therapy and surgical intervention, more than 10% of severe CRSwNP patients show corticosteroid resistance.[17]

There has been much research into the immunologic basis of CRSwNP with the aim of identifying more targeted pharmacologic therapies. Recent studies have shown increased levels of cysLTs and their receptors localized to nasal polyps.[18,19] CysLTs bind to G-protein-coupled receptors to promote localized inflammation, including eosinophil infiltration, mucus production, collagen deposition, and the release of mast cell cytokines.[20] Theoretically, patients with CRSwNP and concomitant asthma or atopy are more responsive to treatment with leukotriene receptor antagonists (LTRAs). This idea is supported by the reduced polyp size and decreased number of systemic eosinophils after montelukast treatment, as eosinophil levels are closely linked to disease severity, CRSwNP, asthma, and allergies.[21,22]

To the best of our knowledge, the expression of GPR17 in the human nasal mucosa has not been previously addressed. In this study, we examined the expression of GPR17 and CysLT1R in nasal tissues. We found that GPR17 and CysLT1R levels were significantly elevated in nasal polyps relative to uncinate tissues from patients with CRSsNP or from control subjects. Next, we analyzed the relationship between GPR17 and CysLT1R levels in nasal polyps. Interestingly, we found a positive association between GPR17 and CysLT1R at both the mRNA and protein levels. Additionally, our previous data and those from other studies have shown that enhanced CysLT1R expression in polyp tissues may play a critical role in the pathogenesis of nasal polyps.[13,18] As GPR17 and CysLT1R are homologous cysLT receptors that are phylogenetically related,[6,7] we speculated that GPR17 and CysLT1R might have somewhat regulatory mechanisms in the pathogenesis of polyp tissues.

Patients with ECRS show a strong possibility of having overlapping mechanisms that cause eosinophilia, and they typically respond poorly to medical management. Accordingly, ECRS is considered a refractory and intractable disease.[23] In the present study, we specifically enrolled an uncontrolled ECRS subpopulation and evaluated GPR17 and CysLT1R levels in polyp tissues. We found a significant difference in the expression of GPR17 and CysLT1R between ECRS and non-ECRS patients. The up-regulated GPR17 and CysLT1R expression in the ECRS subgroup was consistent with our previous report that CysLT1R expression is associated with tissue eosinophilia in CRSwNP patients.[13] In summary, the different GPR17 expression in patients with eosinophilic and non-eosinophilic CRSwNP potentially reflects different pathogenic mechanisms underlying the development of nasal polyps. The findings reported here may contribute to a better understanding of the pathogenic processes involved in these 2 CRSwNP subsets and may aid in designing novel therapeutic strategies that target GPR17 to improve the clinical outcomes of CRSwNP patients.

Conclusion

In summary, our findings indicate that GPR17 and CysLT1R expression is significantly up-regulated in nasal polyps. In addition, we found differential expression of GPR17

between the eosinophilic and non-eosinophilic CRSwNP subsets, which may suggest the existence of distinct pathogenic mechanisms. The correlation between GPR17 and CysLT1R in polyp tissues implies a common regulatory mechanism. The detail of such mechanism remains to be elucidated.

Acknowledgements

This study was supported by the National Natural Science Fund of China under Grant 81470673.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Funding sources for the study

This study was supported by the National Natural Science Fund of China under Grant 81470673.

Authors' contributions

Li and Yang designed the study and wrote the manuscript. Chen supported the writing of the manuscript and the data analysis. Yu participated in the data analysis.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid L, Baroody F, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. Rhinology. 2012; 50:1-12.
2. Hsu J, Peters AT. Pathophysiology of chronic rhinosinusitis with nasal polyp. Am J Rhinol Allergy. 2011; 25:285-90.
3. Akdis CA, Bachert C, Cingi C, Dykewicz MS, Hellings PW, Naclerio RM, et al. Endotypes and phenotypes of chronic rhinosinusitis: A PRACTALL document of the European Academy of Allergy and Clinical Immunology and the American Academy of Allergy, Asthma Immunology. J Allergy Clin Immunol. 2013;131:1479-90.
4. Payne SC, Borish L, Steinke JW. Genetics and phenotyping in chronic sinusitis. J Allergy Clin Immunol. 2011;128:710-20.
5. Chin D, Harvey RJ. Nasal polyposis: an inflammatory condition requiring effective anti-inflammatory treatment. Curr Opin Otolaryngol Head Neck Surg. 2013;21:23-30.
6. Jaconbson KA, Boeynaems JM. P2Y nucleotide receptors: promise of therapeutic applications. Drug Discov Today. 2010;15:570-8.
7. Ciana P, Fumagalli M, Trincavelli ML, Verderio C, Rosa P, Lecca D, et al. The orphan receptor GPR17 identified as a new dual uracil nucleotides/cysteinyl-leukotrienes receptor. EMBO J. 2006;25:4615-27.
8. Lynch KR, O'Neill GP, Liu Q, Im DS, Sawyer N, Metters KM, et al. Characterization of the human cysteinyl leukotriene CysLT1 receptor. Nature. 1999;399:789-93.
9. Saad MA, Abdelsalam RM, Kenawy SA, Attia AS. Montelukast, a cysteinyl leukotriene receptor-1 antagonist protects against hippocampal injury induced by transient global cerebral ischemia and reperfusion in rats. Neurochem Res. 2015;40:139-50.
10. Maekawa A, Xing W, Austen KF, Kanaoka Y. GPR17 regulates immune pulmonary inflammation induced by house dust mites. J Immunol. 2010;185:1846-54.
11. Choi JH, Kim MA, Park HS. An update on the pathogenesis of the upper airways in aspirin-exacerbated respiratory disease. Curr Opin Allergy Clin Immunol. 2014;14:1-6.
12. Steinke JW, Bradley D, Arango P, Crouse CD, Frierson H, Kountakis SE, et al. Cysteinyl leukotriene expression in chronic hyperplastic sinusitis-nasal polyposis: importance to eosinophilia and asthma. J Allergy Clin Immunol. 2003;111:342-9.
13. Wu X, Hong H, Zuo K, Han M, Li J, Wen W, et al. Expression of leukotriene and its receptors in eosinophilic chronic rhinosinusitis with nasal polyps. Int Forum Allergy Rhinol. 2016;6:75-81.
14. Wen W, Liu W, Zhang L, Bai J, Fan Y, Xia W, et al. Increased neutrophilia in nasal polyps reduces the response to oral corticosteroid therapy. J Allergy Clin Immunol. 2012;129:1522-8.
15. Mullol J, Obando A, Pujols L, Alobid I. Corticosteroid treatment in chronic rhinosinusitis: the possibilities and limits. Immunol Allergy Clin North Am. 2009;29:657-68.
16. Nonaka M, Tanaka Y, Pawankar R, Yoshihara T. Orally inhaled fluticasone propionate improved chronic rhinosinusitis with co-asthma: report of a case. Asian Pac J Allergy Immunol. 2013;31(1):84-87.
17. Hellings PW, Fokkens WJ, Akdis C, Bachert C, Cingi C, Dietz de Loos D, et al. Uncontrolled allergic rhinitis and chronic rhinosinusitis: where do we stand today? Allergy. 2013;68:1-7.
18. Chao SS, Graham SM, Brown CL, Kline JN, Hussain I. Cysteinyl leukotriene 1 receptor expression in nasal polyps. Ann Otol. 2006;115:394-7.
19. Schäper C, Noga O, Koch B, Ewert R, Felix SB, Gläser S, et al. Anti-inflammatory properties of montelukast, a leukotriene receptor antagonist in patients with asthma and nasal polyposis. J Investig Allergol Clin Immunol. 2011;21:51-8.
20. Holgate ST, Peters-Golden M, Panettieri RA, Henderson WR Jr. Roles of cysteinyl leukotrienes in airway inflammation, smooth muscle function, and remodeling. J Allergy Clin Immunol. 2003;111:S18-34.
21. Schmid C, Habermann W, Braun H, Gugatschka M, Oriel BS, Smietana JA, et al. Released intranasal eosinophilic major basic protein as a diagnostic for polypoid chronic rhinosinusitis. Otolaryngol Head Neck Surg. 2010;143:386-91.
22. Ahmadiafshar A, Taghiloo D, Esmailzadeh A, Falakaflaki B. Nasal eosinophilia as a marker for allergic rhinitis: a controlled study of 50 patients. Ear Nose Throat J. 2012;91:122-4.
23. Ishinaga H, Shah SA, Sakaida H, Takeuchi K. The role of transforming growth factor-α on mucin overproduction in eosinophilic chronic rhinosinusitis. Pharmacology. 2011;88:302-8.

APJAI
Asian Pacific Journal of
Allergy and Immunology

ORIGINAL ARTICLE

Solenopsis geminata (tropical fire ant) anaphylaxis among Thai patients: its allergens and specific IgE-reactivity

Rutcharin Potiwat,[1] Sasipa Tanyaratsrisakul,[2] Santi Maneewatchararangsri,[2] Wiparat Manuyakorn,[3] Ticha Rerkpattanapipat,[4] Yudthana Samung,[1] Chukiat Sirivichayakul,[5] Wanpen Chaicumpa,[6] and Raweerat Sitcharungsi[5]

Abstract

Background: Specific IgE against *Solenopsis invicta* (imported fire ant) remains the current diagnostic tool for allergy to ants worldwide. However, *S. invicta* may not be the only cause of ant anaphylaxis in Thai patients.

Objective: To characterize ant species causing anaphylaxis in Thai patients and to test allergenic reactivity to whole body extracts (WBE) of *S. geminata* (tropical fire ants) in patients with evidence of IgE-mediated ant anaphylaxis.

Methods: Thirty-two patients with ant anaphylaxis were identified. The causative ants collected by the patients were subjected to species identification. Twelve patients with ant anaphylaxis and showed positive skin test or serum specific IgE to *S. invicta* and 14 control subjects were recruited. Whole body extraction from *S. geminata* was performed for protein characterization using SDS-PAGE and protein staining. IgE-immunoblotting and ELISA-specific IgE binding assays were performed on patients' sera and compared with controls.

Results: Of 32 patients with ant anaphylaxis, the most common causative ant identified was *S. geminata* (37.5%). Western blot analysis of crude *S. geminata* revealed 13 refined protein components that bound to patients' serum IgE. Three major allergens with molecular masses of 26, 55 and 75 kDa were identified. All 12 patients gave positive results for specific IgE to *S. geminata* with statistically significant higher absorbance units of 0.390 ± 0.044, compared to healthy control group (0.121 ± 0.010), $P < 0.01$.

Conclusions: *S. geminata* is identified as the most common causative ant anaphylaxis in Thai patients. Its WBE comprises of 13 IgE-binding components and 3 major allergens (26, 55 and 75 kDa), which supported possible IgE-mediated mechanism.

Keywords: Fire ant, ant allergy, *Solenopsis geminata*, *Solenopsis invicta*, specific IgE-ELISA

From:
[1] Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand
[2] Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand
[3] Department of Pediatrics, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand
[4] Department of Medicine, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand
[5] Department of Tropical Pediatrics, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand
[6] Center of Excellence on Therapeutic Proteins and Antibody Engineering, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand

Corresponding author:
Raweerat Sitcharungsi
Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand
Email: raweeratsitcharungsi@gmail.com

Abbreviations:
kDa = kilodalton
ELISA = Enzyme-linked immunosorbent assay
WBE = Whole body extract
IFA = imported fire ant
TFA = tropical fire ant

Introduction

Ant hypersensitivity can be fatal, owing to a systemic reaction or anaphylaxis.[1-3] Fire ant anaphylaxis is a serious problem worldwide with reports on fatalites.[2,4] Imported fire ant (IFA), in particular *Solenopsis invicta* (red imported fire ant) and *S. richteri* (black imported fire ant) are well-known for causing life-threatening allergic reactions.[5] The risk of

IFA stings in people living in endemic areas is as high as 30%-60%,[5,6] with a 0.16–16% risk of severe reactions.[7-9] In addition to *Solenopsis* species, *Formica*, *Myrmecia*, *Tetramorium*, *Pogonomyrmex*, *Pachycondyla*, *Odontomachus*, *Rhytidoponera*, *Pseudomyrmex* and *Hypoponera* are ant genera that have also been reported to cause serious hypersensitivity reactions.[10] *Solenopsis geminata* has been reported to be an important stinging ant species causing anaphylaxis in many Asian islands, including Indonesia and Taiwan.[11] Imported fire ants were introduced from South America and spread to Southeastern regions of the United States from 1920 onwards.[12] Currently, IFAs are endemic in some areas of Asia, such as China and Hong Kong, and some parts of Australia.[12]

In Thailand, there are as many as 247 species of ants belonging to nine subfamilies.[13] However, IFAs have not been detected in Thailand. The most common fire ant species found in Thailand is *S. geminata* (tropical fire ant, TFA), but the most common causes of ant anaphylaxis include both *S. geminata* and *Tetraponera* spp.[2,14] However, the confirmation of ant anaphylaxis in suspected patients is currently based on a skin test or specific IgE against *S. invicta* whole body extract (WBE), the only available commercial tests for ant allergy.[7] There have been limited data on cross-reactivity among ant species and only a few studies have reported cross-reactivity between the venom of *Solenopsis* spp.[15,16] and *Pogonomyrmex* spp.[2] Interestingly, a proportion of Thai patients with an obvious history of ant anaphylaxis had negative skin tests and specific IgE against IFAs. This indicated a lack of specific IgE or skin testing reagent to relevant local allergens, as the mechanism of hypersensitivity reaction to ant sting in Thai patients was supposed to be similar to that of IFA sting.

We aimed to identify the *S. geminata* allergen by preparing crude WBE and study of IgE-binding from patients' sera with evidence of IgE-mediated ant anaphylaxis to *S. invicta*.

Methods

Patients

Thirty-two children and adults with a history of ant anaphylaxis that were seen at allergy clinics at the Faculty of Medicine, Ramathibodi Hospital and the Hospital for Tropical Diseases, and the Faculty of Tropical Medicine, Mahidol University, during 2012–2014, were identified. The inclusion criteria were patients who had a history of anaphylaxis from ant stings and positive results for specific IgE or skin tests using *S. invicta* WBE. The level of specific IgE to IFAs of > 0.35 kAU/L was interpreted as a positive result. The reaction of skin prick test (SPT) was considered positive when a wheal diameter was at least 2 mm larger than negative control with surrounding erythema at 15 minutes. All positive SPT must be at least 3 mm in diameter size. Patients with negative SPT, would proceed to intradermal test, which was considered positive when a wheal diameter was increased at least 3 mm from injection papules with pruritus or surrounding flare after 20 minutes.[17] These are the current methods for diagnosing fire ant anaphylaxis in Thailand because specific investigations for other type of ant, including TFA are not available. Baseline data, including age, gender, underlying diseases, history of stinging hypersensitivity and clinical manifestations, were collected.

Control subjects

Fourteen control subjects were recruited at the Faculty of Tropical Medicine, Mahidol University after obtaining written informed consents. They were healthy volunteers who had history of a fire ant sting without any symptoms or only a small local reaction.

Causative ant identification

All patients were asked to bring the suspected causative ants from the areas where they were stung to the hospital for species identification. The ant species were identified by a taxonomist from The Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University. Because *S. geminata* was found to be the most common ant causing anaphylaxis in the patients, the surveillance teams went to the endemic areas to collect ant samples by direct searching.

Collection of tropical fire ants

Tropical fire ant colonies were collected from patient's houses and were placed into plastic containers with a capacity of $3.14 \times (6.4)^2 \times 9.4$ cm^3, covered with nylon net for air flow. Meat and 10% sugar solution were provided in each container in a cup during transfer of the sample to the laboratory. The ants were washed three times in phosphate buffered saline (PBS; 140 mM NaCl, 10 mM phosphate buffer and 3 mM KCl, pH 7.4 at 25°C; MERCK Millipore), then stored at −80°C before the preparation of crude WBE solution.

Preparation of tropical fire ant allergen

Crude fire ant allergen was prepared from the collected *S. geminata*. Briefly, frozen fire ants were cryogenically grinded in liquid nitrogen until a powder of material appeared. Then, the soluble contents were dissolved in sterile normal saline (NSS) at 4°C for 2 h. Debris was removed by centrifugation at 10,000 g (25°C for 15 min) and the supernatant containing tropical fire ant extract was collected. The total protein concentration was determined using a Pierce™ BCA protein assay kit and bovine serum albumin as the standard (Thermo Fisher Scientific, MA, USA). Allergen was verified by SDS-PAGE and colloidal Coomassie brilliant blue G-250 staining. The extract was stored at -70°C until use. The contamination of *S. geminata* extract was also minimized by using protein-free plastic ware, and sterilizing the cleaned mortar and grinder to prevent the contamination of in-house allergen extract.

SDS-PAGE

Allergens from TFAs-*S. geminata* and IFAs-*S. Invicta* (ALK -Abello Inc., USA), were separated by 13% SDS-PAGE under reducing conditions (20 µg per well) using a Mini-PROTEAN® Electrophoresis System (Bio-Rad, USA). SDS-PAGE was carried out at 20 mAmp/gel for 45 minutes. Then, separated proteins were stained by colloidal Coomassie brilliant blue G-250, followed by de-staining until the background was clear.

IgE-immunoblot

Tropical and imported fire ant allergens were subjected to 13% SDS-PAGE as described above and then electro-transferred onto PVDF membrane (PALL Corporation, PA, USA).

The membrane was blocked with blocking buffer (5% skim milk in PBS, pH 7.4) for 1 h at 25°C. Then, it was incubated with a 1:100 dilution of individual serum samples in blocking buffer for 2 h at 25°C. Thereafter, it was washed five times, each time for five min with washing buffer (0.05% Tween-20 PBS; PBS-T). Finally, the membrane was incubated with horseradish peroxidase-conjugated mouse anti-human IgE antibody (Southern Biotech, USA) at a dilution of 1:4,000 for 1 h at 25°C, followed by washing as described above. Fire ant allergen-IgE antibody reactive bands were detected by chemiluminescent substrate (Thermo Fisher Scientific (Pierce), USA) and an ImageQuant LAS 4000 mini imager (GE Healthcare Bio-Sciences AB, Sweden). Three experiments were carried out in 12 patients and 14 healthy controls.

IgE-ELISA assay

ELISA assays were performed to investigate *S. geminata* IgE reactivity in patients' sera. First, TFA allergen (500 ng/well in PBS pH 7.4) was added to an ELISA plate (ExtraGene Inc., Taiwan) and incubated at 4°C overnight. Unbound allergens were washed off with 200 µl of washing buffer (PBS-T) twice, then the wells were blocked with 150 µl of blocking reagent (5% skim milk in PBS) at 37°C for 1 h. After washing, 100 µl of 1:100-diluted serum or blocking reagent alone was added to each well and the plate was incubated at 37°C for 1 h. After washing the ELISA plate five times, 100 µl of a 1:4,000-dilution of horseradish peroxidase-conjugated mouse anti-human IgE secondary antibody (Southern Biotech) were added to each ELISA well and the plate was incubated as described above. After washing, 100 µl of 1-Step™ Ultra TMB substrate (Thermo Fisher Scientific (Pierce), USA) was added to each well. After 15 min incubation, 100 µl of 2 N sulfuric acid was added to each well. The optical density (OD 450 nm) of IgE-ELISA was measured using a microplate reader (Bio-Tek Instruments, VT, USA) at 450 nm (OD_{450nm}). The experiment was performed in duplicate on three independent occasions. For *S. invicta*, the IgE binding was very low and not much different from the background control. This might be the low concentration of *S. invicta* protein from commercial extract and the high viscosity of piperidine solvent, which may affect protein coating efficacy. We thus reported only western blot analysis of *S.invicta*.

Statistical analyses

Median, interquartile range (IQR) or frequency percentage distributions were calculated according to the type of variables for the demographic data. IgE reactivity results analysis was performed by an unpaired t-test using the software GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA).

Results

Ant species identification and patients' clinical characteristics

Thirty-two patients with a history of anaphylaxis from ant stings were identified. The suspected causative ants brought in by the patients were identified by the taxonomist and were attributed to the following genera: 12 *S. geminata* (37.5%), 10 *Tetraponera* spp. (31.3%), 2 *Odontoponera* spp. (6.25%), 1 *Pachycondyla* spp. and 1 *Monomorium* spp. Six patients (18.75%) were unable to provide a sample of the causative ants but suspected that they were stung by *S. geminata*.

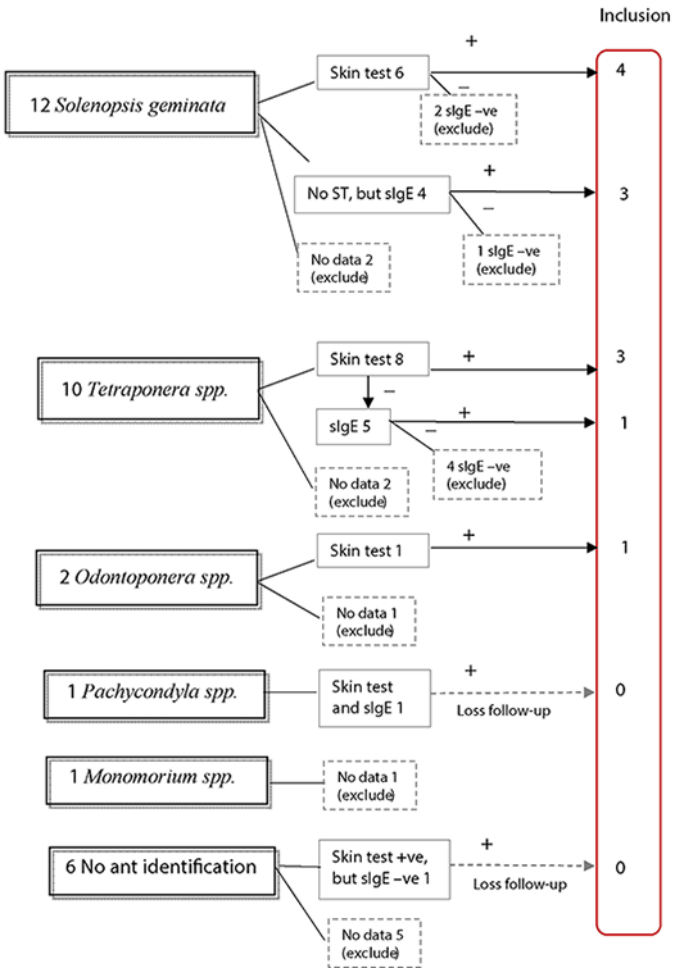


Figure 1. Recruitment diagram of 32 patients.
sIgE: specific IgE to *S.invicta*

Figure 1 showed recruitment diagram of 32 patients. 7 of 12 patients stung by *S.geminata*, 4 of 10 stung by *Tetraponera spp.*, and 1/2 stung by *Odontopenera* with positive tests to *S.invicta* were included in the study. Unfortunately, we have 2 positive patients who loss to follow-up (1 stung by *Pachycondyla* and another with no ant identification). Testing data were missing in 11 patients. Overall skin test to *S.invicta* was performed in 17 patients: positive in 10 and negative in 7 patients. And specific IgE to *S.invicta* was done in 13 patients: positive in 5 and negative in 8 patients.

A total of 12 patients who showed positive results with the specific IgE or skin test using *S. invicta* WBE were recruited into the patient group and 14 healthy volunteers were recruited into the control group. The clinical characteristics of the patients and control subjects are shown in **Table 1**. Seven patients (7/12; 58.3%) were female with a median age (IQR) of 12.5 (5–63) years, while 11 of 14 (78.6%) control subjects were female, and a median (range) age of 28 (23–39) years. Nine patients had a history of atopic diseases including allergic rhinitis (50%), allergic rhinoconjunctivitis (8.3%) and asthma (33.3%). All atopic diseases were under-controlled during the study. Two subjects (14.3%) in the control group had a history of allergic rhinitis.

Table 1. Clinical characteristics of 15 patients with fire ant anaphylaxis and 14 control subjects

Subject No.	Gender	Age (yr)	Atopy	Symptoms	Causative ant from ant identification	Serum gEIFA	Skin test IFA
P1	M	8	AR, ASTHMA	S, RS	*S. geminata*	9.33	+
P2	F	10	ARC	S, RS, GI	*S. geminata*	1.57	+
P3	F	37	N	S, RS	*S. geminata*	<0.35	+
P4	F	27	AR	S, RS, CVS	*S. geminata*	13.4	+
P5	M	12	AR, ASTHMA	S, RS	*S. geminata*	11.0	ND
P6	M	7	ASTHMA	S, CVS	*Odontoponera denticulata*	22.4	+
P7	M	5	ASTHMA	S, RS	*Tetraponera rufoniara*	0.74	-
P8	F	63	N	S, CVS	*Tetraponera rufoniara*	<0.35	+
P9	M	16	AR	S, RS	*Tetraponera rufoniara*	5.62	+
P10	F	13	N	S, RS, CVS	*Tetraponera rufoniara*	21.6	+
P11	F	11	AR	S, RS	*S. geminata*	1.84	ND
P12	F	18	AR	S, RS	*S. geminata*	3.37	ND
C1	F	29	AR	N	ND	<0.35	ND
C2	F	23	N	N	ND	<0.35	ND
C3	F	24	N	N	ND	<0.35	ND
C4	F	39	N	N	ND	<0.35	ND
C5	M	32	N	N	ND	<0.35	ND
C6	F	36	N	N	ND	<0.35	ND
C7	F	25	N	N	ND	<0.35	ND
C8	F	27	N	N	ND	<0.35	ND
C9	M	28	N	N	ND	<0.35	ND
C10	F	28	N	N	ND	<0.35	ND
C11	M	27	N	N	ND	<0.35	ND
C12	F	37	N	N	ND	<0.35	ND
C13	F	27	N	N	ND	<0.35	ND
C14	F	29	AR	N	ND	<0.35	ND

P, patient; C, control; M, male; F, female; AR, allergic rhinitis; ARC, allergic rhino-conjunctivitis; S, skin manifestations such as urticaria, angioedema, and swelling of eyes and mouth; RS, symptoms of respiratory system such as wheeze and bronchospasm; CVS, symptoms of cardiovascular system such as low blood pressure, shock; GI, gastrointestinal symptoms such as vomiting; IFA, imported fire ant extract; N, none; +, positive; -, negative; ND, not determined.

The level of serum-specific IgE to IFAs was positive in 10 patients (83.3%), while the skin test was positive in 8 of 9 patients (88.9%). All control subjects gave negative results for serum-specific IgE to IFAs.

All patients reported severe systemic reactions after a clearly identified ant sting. The most frequently involved symptom was urticaria, which was reported in all patients. Ten patients had respiratory symptoms such as wheezing, dyspnea and a spasmodic cough. Three patients had syncope, two patients suffered shock and one patient had gastrointestinal symptoms, which included vomiting. Most patients had been stung only once. Finally, among 12 patients recruited, 7 were stung by *S.geminata*, 4 by *Tetraponera rufoniara*, and 1 by *Odontoponera denticulata*.

Fire ant allergen profile and specific IgE reactivity

The WBE of TFA allergen was prepared from crude extract of *S. geminata*. Its protein contents showed a significantly different profile on an SDS-PAGE gel compared to IFA allergen (*S. invicta*). While, TFA extract revealed more than 20 bands of protein ranging from 10 to 200 kDa, IFA allergens appeared to have only two major bands between 25 and 35 kDa. (**Figure 2**). To verify background binding, mock allergen extract without fire ant was included in western blotting and nothing was detected with serum from two randomly selected patients (data not shown).

Determination of specific IgE against S. geminata and S. invicta

Specific IgE to *S. geminata* and *S. invicta* was detected by western blot analysis and 13 reactive bands were detected in patients' serum (**Figure 3** and **Table 2**). The common IgE -specific reactive bands of *S. geminata* (presented in >80% of ant allergic patients' serum) had estimated molecular masses of 26, 55 and 75 kDa, which showed reactivity in 100%, 83.3% and 91.7% of patients, respectively. (Arrows, **Figure 3**). More than 50% of patients also had IgE-reactive band to *S.geminata*'s protein at molecular weight of 85 and 95 kDa. (**Table 2**) For *S. invicta* allergen, only weak IgE reactive bands at 26 and 55 kDa were identified. The serum of control subjects (n=14) did not show any reactive bands for either *S. geminata* or *S. invicta*

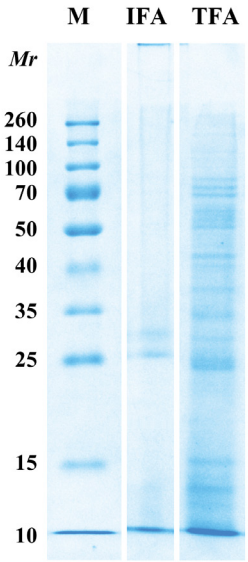


Figure 2. SDS-PAGE showing protein compositions of imported fire ant (IFA) and tropical fire ant (TFA) extracts according to relative molecular mass (Mr).
Mr: relative molecular masses ranging from 10 to 260; M: molecular weight marker

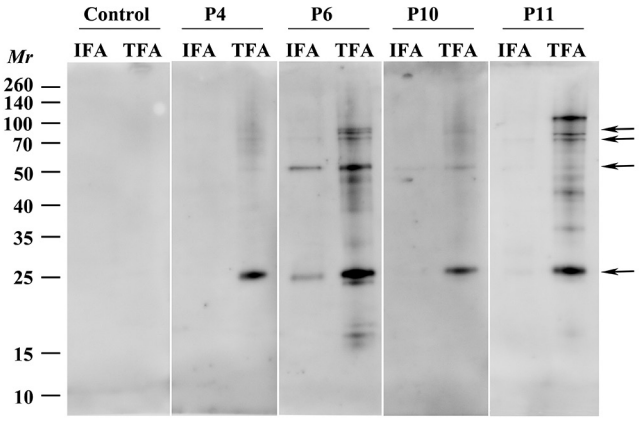


Figure 3. Immunoblot of IgE-reactive components of *S. invicta* (IFA) and *S. geminata* (TFA) extracts among patients and healthy controls
IgE-reactive patterns of 4 allergic patients (P4, P6, P10 and P11) and one healthy control are shown. Arrows indicate common IgE-reactive allergens of tropical fire ants that reacted with more than 80% of allergic patients. The healthy control did not show any IgE-reactive bands.
Mr: relative molecular masses of the proteins; IFA: imported fire ant; TFA: tropical fire ant

Table 2. The IgE reactivity profile of tropical fire ant allergens in patients with evidence of IgE-mediated to *S. invicta*

MW (kDa)	Patient number												% of patients reacted to each reactive band
	1	2	3	4	5	6	7	8	9	10	11	12	
115	●	●			●						●		33.3
95		●			●	●	●	●	●	●			58.3
85		●			●	●	●	●	●		●		58.3
75	●	●	●	●	●	●	●	●	●		●	●	91.7
55	●	●	●	●	●	●	●		●	●	●		83.3
48			●			●					●	●	33.3
44		●									●	●	25.0
38		●	●		●	●							33.3
36											●		8.3
33						●		●					16.7
26	●	●	●	●	●	●	●	●	●	●	●	●	100.0
24			●			●			●				25.0
17			●			●		●			●		33.3

Filled circles (●) represent protein bands that reacted with serum IgE from each patient, as determined by western blot analysis.
MW: molecular weight of IgE-binding protein, kDa: kilodalton

allergens, as shown in **Figure 3**.

Specific IgE ELISA reactivity to S. geminata allergen

The specific IgE reactivity to TFA allergen was determined by ELISA using patients' and control subjects' sera at a dilution of 1:100. The absorbance unit of IgE reactivity was significantly higher in patients compared with healthy controls (0.390 ± 0.044 and 0.121 ± 0.010, respectively; P value <0.01), with a greater than 99% confidence interval (**Figure 4**). Using the cut-off value of 0.151 (mean of control + 3×standard deviation (3SD)), 100% (12/12) of patients gave a positive IgE-specific result to the crude extract of TFAs.

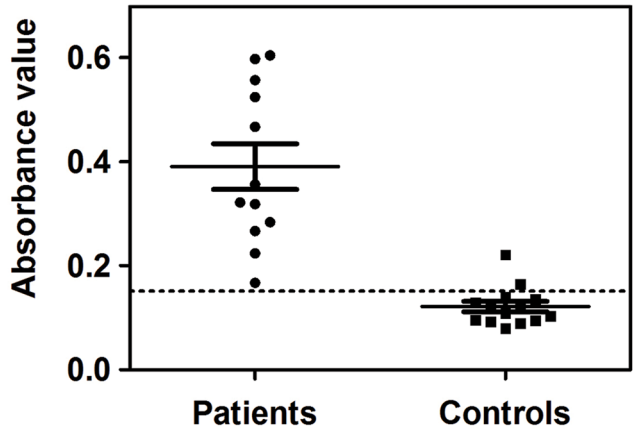


Figure 4. Specific IgE ELISA reactivity to *S. geminata* allergens among patients and healthy controls
The specific serum-IgE binding reactivity is shown at an absorbance value (OD 405 nm) for individual subjects. Dashed line indicates the cut-off value at 0.151 (mean of control + 3SD). The data are averages from three independent experiments.

Discussion

Our study is the first and the largest case series of ant anaphylaxis in Thai population. It is the first report to demonstrate that IgE antibody from *S. invicta* allergic patients with a history of ant anaphylaxis, recognized allergenic components present in *S. geminata* allergen extracted in-house, differentially from that of IFA allergen.

All patients in our study displayed anaphylaxis, with 100% showing cutaneous manifestations and 83% showing respiratory symptoms. One limitation of our study was the small number of patients since we enrolled only patients who presented with severe allergic reactions referred to the Medical Centers in Bangkok, and most therefore lived in Bangkok or in nearby provinces. Some cases of ant anaphylaxis that resulted in death in the southern and northern parts of Thailand would have been reported in the newspaper, but the provision of medical details was limited. From 32 patients identified, only 12 of these were recruited because of the requirement of evidence of specific IgE to fire ant, and the limitation of available test allergen. We did not include patients who had anaphylaxis to *S.geminata*, but had negative testing to *S.invicta*, and thus we could have missed some cases of mono-sensitized to *S.geminata*. Despite the possibility of uncertainty regarding the species of the causative ants because some patients might not be sure that they had brought in the correct species. We demonstrated that our patients with presence of specific IgE to *S.invicta*, actually reacted to various type of ants, that were *S. geminata*, *Tetraponeera rufoniara*, *Odontoponera denticulata* and *Pachychondyla spp*. This finding suggested the possibility of cross-reactivity between *S.invicta* and other genera of ants, other than *S.geminata*.

Of the 12 patients included in our study, positivity of specific IgE levels to IFA extract was found in 83.3% (10/12), while skin test was positive in 88.9% (8/9). Two patients (16.7%) who had negative results of specific IgE to IFA, both had positive skin test. A practice parameters for stinging insect hypersensitivity had revealed that 20% of patients with positive skin test results for hymenoptera venom allergy showed negative results for serum-specific IgE.[17] Another study demonstrated that serum *S. invicta*-specific IgE had lower sensitivity than the skin test for the diagnosis of fire ant allergy.[18] These evidences were all consistent with our findings. However, our finding might also indicate the limitation of using *S. invicta*-specific IgE for the diagnosis of ant hypersensitivity possibly caused by *S. geminata* and *Tetraponera spp*. among the Thai population.

In the current study, majority of patients' serum IgE bound to the allergens of WBE of crude *S. geminata*, and it suggested that hypersensitivity reaction to *S. geminata* could be a type I, IgE-mediated mechanism.

Using ELISA, all 12 patients displayed serum IgE specific to *S. geminata* allergens, and significantly higher IgE reactivity was observed in the patients compared with the healthy controls. This might indicate that TFA extract is more sensitive than IFA extract for the identification and diagnosis of patients suffering from ant hypersensitivity in Thailand. However, this was limited by the findings that two healthy subjects showed slightly higher specific IgE levels, eventhough their IgE-bindings were not detected by western blot analysis. Thus, false positive of specific IgE-ELISA system could be suspected with the rate of 14.3% (2/14). Anyway, the specificity of crude WBE of TFA was supported by the western blot data as well, in which serum IgE from all patients reacted to various TFA proteins, whereas none of the healthy controls showed reactivity. Although we found more than 10 proteins recognized by the IgE from patients' sera, the proteins with relative molecular weights of 26, 55 and 75 kDa showed more than 80% recognition, which suggested that these three proteins might be the major allergens of TFAs.

Surprisingly, only weak IgE binding to *S. invicta* extract was observed in 33% of patients by immunoblotting, despite that majority of patients had specific IgE level to *S.invicta* at baseline. All patients in our study received IFA immunotherapy, which might explain a decrease the serum IgE binding against *S. invicta*, as detected by immunoblotting as well as very low binding of IgE to *S.invicta* allergens in IgE-ELISA system. Moreover, the serum from patients at the time of positive test to specific IgE to *S. invicta* were not available, and blood sample to perform immunoblotting were drawn separately. However, the major reactive proteins were found to have similar molecular weights of 26 and 55 kDa to the major allergens of *S. geminata*. This indicated the possibility of cross-reactivity of patients' serum-specific IgE, with IgE induced by *S. geminata* allergens cross-reacting to that of *S. invicta*.

Owning to the high number of protein bands of crude WBE of TFA, the contamination of *S. geminata* extract was also concerned. We have multiple steps in preparation of crude extract to ensure the purification: washing the collected ant with PBS before use, using protein-free plastic ware, and sterilizing the cleaned mortar and grinder. Moreover, we have performed western blot analysis of mock allergen extract without fire ant, and nothing was detected with serum from two randomly selected patients.

The venom of IFAs is unique because it has a low protein content and a high concentration of piperidine toxin that consists of 90–95% water-soluble alkaloids, 2-methyl-6-alkylpiperidines.[7,19] Currently, four important allergens have been isolated and characterized from *S. invicta* including Sol i 1 (37 kDa), Sol i 2 (26 kDa), Sol i 3 (24 kDa) and Sol i 4 (13 kDa).[19-21]

The Sol i 4 allergen has 35% identity with the Sol i 2 allergen but does not immunologically cross-react.[22] Similarly, *S. geminata* venom harbors four allergenic proteins designated Sol gem 1, Sol gem 2, Sol gem 3 and Sol gem 4. After evaluating Sol gem 2 under non-reducing and reducing conditions, its native form was identified as a dimer with molecular weights of 28 and 15 kDa and allergenic properties similar to Sol i 2 of *S. invicta*.[23]

A previous study demonstrated that the standard dosage of 0.5 ml of 1:100 wt/vol of *S. invicta* used as immunotherapy for ant anaphylaxis Thai patients demonstrated only 60% efficacy for preventing a field ant re-sting. However, after increasing the dosage to 0.5 ml of 1:50 wt/vol of *S. invicta*, the efficacy increased to 100%.[24] Since there is no *S. invicta* in Thailand and the majority of ant in Thailand is *S. geminata* and *Tetraponera spp*. This study may be an indirect evidence of the possibly cross-reactivity between *S. invicta* and other ants found in Thailand, particularly *S. geminata* and *Tetraponera spp*. on clinical outcome.

Information found in this study may be crucial for future studies using recombinant major allergens from TFAs as diagnostic and, possibly, therapeutic agents for ant allergic patients. The inclusion of patients from various regions in Thailand and patients negative for specific IgE or skin tests to *S. invicta* is important to confirm this hypothesis. In patients who had negative testing to both TFA extract and *S.invicta*, *Tetraponera spp*. seems to be another important ant causing anaphylaxis in Thai patients and worth studying. The properties and functions of targeted allergens should be explored to design effective diagnostic and therapeutic strategies. Whole body extractions of TFAs are producible as in-house allergens and might be applied for skin prick tests or immunotherapy in the future.

Conclusion

S. geminata was identified as the most common causative ant in Thai patients with ant anaphylaxis. Whole body extraction of *S. geminata*, prepared in-house for allergenic studies, revealed 13 IgE-binding components using patients' sera, with three major allergens (26, 55 and 75 kDa). Moreover, all studied patients harbored specific IgE antibody recognizing the allergenic components present in *S. geminata* supported the possible IgE-mediated mechanism to *S. geminata*. The 26 and 55 kDa allergens may induce the production of cross-reactive IgE that can recognize allergens from both *S. geminata* and *S. invicta*.

Acknowledgements

We are grateful to all of the patients and their families for participating in this study. We thank Clin. Prof. Suwat Benjaponpitak for help and advice on the research methodology, Asst. Prof. Saranath Lawpoolsri Niyom for advice on statistical analysis, and Assoc. Prof. Dr. Decha Wiwatwittaya for the ant specimen collection. We thank Ms. Pataporn Wisetsing and Ms. Pudtamaporn Tiangthum for preparing the serum from all of the patients. We also thank the Center of Research Excellence on Therapeutic Proteins and Antibody Engineering, Faculty of Medicine Siriraj Hospital, for providing some reagents. This study was supported by the Thailand Research Fund (TRF, grant number MRG5580064) and the Faculty of Tropical Medicine, Mahidol University. The authors declare that there is no conflict of interest regarding the publication of this paper.

References

1. Tankersley MS, Ledford DK. Stinging insect allergy: state of the art 2015. J Allergy Clin Immunol Pract. 2015;3:315-22.
2. Potiwat R, Sitcharungsi R. Ant allergens and hypersensitivity reactions in response to ant stings. Asian Pac J Allergy Immunol. 2015;33:267-75.
3. Srisong H, Daduang S, Lopata AL. Current advances in ant venom proteins causing hypersensitivity reactions in the Asia-Pacific region. Mol Immunol. 2016;69:24-32.
4. Haddad Junior V, Larsson CE. Anaphylaxis caused by stings from the Solenopsis invicta, lava-pés ant or red imported fire ant. Anais Brasileiros de Dermatologia. 2015;90:22-5.
5. deShazo RD, Butcher BT, Banks WA. Reactions to the stings of the imported fire ant. N Engl J Med. 1990;323:462-6.
6. deShazo RD, Williams DF, Moak ES. Fire ant attacks on residents in health care facilities: a report of two cases. Ann Intern Med. 1999;131:424-9.
7. Stafford CT. Hypersensitivity to fire ant venom. Ann Allergy Asthma Immunol. 1996;77:87-95.
8. Adams CT, Lofgren CS. Red imported fire ants (Hymenoptera: Formicidae): frequency of sting attacks on residents of Sumter County, Georgia. J Med Entomol. 1981;18:378-82.
9. Triplett RF. The imported fire ant: health hazard or nuisance? South Med J. 1976;69:258-9.
10. Klotz JH, deShazo RD, Pinnas JL, Frishman AM, Schmidt JO, Suiter DR, et al. Adverse reactions to ants other than imported fire ants. Ann Allergy Asthma Immunol. 2005;95:418-25.
11. Hoffman DR. Ant venoms. Curr Opin Allergy Clin Immunol. 2010;10: 342-6.
12. La Shell MS, Calabria CW, Quinn JM. Imported fire ant field reaction and immunotherapy safety characteristics: the IFACS study. J Allergy Clin Immunol. 2010;125:1294-9.
13. Jaitrong W, Nabhitabhata J. A list of known ant species of Thailand (Formicidae: Hymenoptera). Thailand Natural History Museum J. 2005;1(1):9-54.
14. Puranitee P, Vilaiyuk S, Kamchaisatian W, Manuyakorn W, Teawsomboonkit W, Sasisakunporn C, et al. Reported ant re-sting reaction in Thai children with ant anaphylaxis receiving immunotherapy. J Allergy Clin Immunol. 2012;115:AB228.
15. Hoffman DR, Smith AM, Schmidt M, Moffitt JE, Guralnick M. Allergens in Hymenoptera venom. XXII. Comparison of venoms from two species of imported fire ants, Solenopsis invicta and richteri. J Allergy Clin Immunol. 1990;85:988-96.
16. Pinnas JL, Strunk RC, Wang TM, Thompson HC. Harvester ant sensitivity: In vitro and in vivo studies using whole body extracts and venom. J Allergy Clin Immunol. 1977;59:10-6.
17. Golden DBK, Moffitt J, Nicklas RA, Freeman T, Graft DF, Reisman RE, et al. Stinging insect hypersensitivity: A practice parameter update 2011. J Allergy Clin Immunol. 2011;127:852-4.e1-23.

18. Stafford CT, Moffitt JE, Bunker-Soler A, Hoffman DR, Thompson WO. Comparison of in vivo and in vitro tests in the diagnosis of imported fire ant sting allergy. Ann Allergy. 1990;64:368-72.
19. Hoffman DR. Reactions to less common species of fire ants. J Allergy Clin Immunol. 1997;100:679-83.
20. Hoffman DR. Allergens in Hymenoptera venom XXIV: the amino acid sequences of imported fire ant venom allergens Sol i II, Sol i III, and Sol i IV. J Allergy Clin Immunol. 1993;91:71-8.
21. Hoffman DR, Dove DE, Jacobson RS. Allergens in Hymenoptera venom: XX. Isolation of four allergens from imported fire ant (Solenopsis invicta) venom. J Allergy Clin Immunol. 1988;82:818-27.
22. Hoffman DR. Fire ant venom allergy. Allergy. 1995;50:535-44.
23. Sukprasert S, Uawonggul N, Jamjanya T, Thammasirirak S, Daduang J, Daduang S. Characterization of the allergen Sol gem 2 from the fire ant venom, Solenopsis geminata. J Venom Anim Toxins Trop Dis. 2012;18: 325-34.
24. Manuyakorn W, Benjaponpitak S, Kamchaisatian W, Sasisakulporn C, Jotikasthira W. Safety and efficacy of ant rush immunotherapy in children. Asian Pac J Allergy Immunol. Forthcoming 2016.

APJAI
Asian Pacific Journal of
Allergy and Immunology

CASE REPORT

Recurrent anaphylaxis in patient allergic to eggplant – a Lipid transfer protein (LTP) syndrome

Natalia Ukleja-Sokołowska,[1] Ewa Gawrońska-Ukleja,[1] Magdalena Żbikowska-Gotz,[1] Łukasz Sokołowski,[2] Zbigniew Bartuzi[1]

Abstract

Eggplant allergy is rare and most of the previously described reactions were mild. In this case report, we present an interesting case of a 27-year-old male who experienced symptoms of anaphylaxis (shortness of breath, and swelling of the face, lips and tongue, which was followed by hypotension, tachycardia of 140/min and a sudden loss of consciousness) several minutes after eating a dish containing backed eggplant. Previously, the patient had experienced symptoms of allergy after eating different types of food, such as salad and chicken in spices. The symptoms were enhanced by co-factors. They were heterogeneous, ranging from oral symptoms to anaphylactic shock.

During the diagnostic pathway, skin prick tests (SPTs) were positive to grass and cat. Prick by prick skin tests were positive for eggplant, both cooked and fresh. In ImmunoCap ISAC, IgE specific to rPhl p 1 (1.7 ISU-E), rCan f 5 (1,2 ISU-E), Fel d 1 (9.6 ISU-E) and LTPs - nJug r 3 (0.5 ISU-E), rPru p 3 (0.6 ISU-E), rPla a 3 (1.3 ISU-E) were found.

Based on the clinical pattern of the disease and the results of component resolved diagnosis, we suspect that this complex case of anaphylaxis may be gathered with LTP syndrome.

Keywords: anaphylaxis, eggplant, allergy, component resolved diagnosis, lipid transfer protein

From:
[1] Department and Clinic of Allergology, Clinical Immunology and Internal Diseases, Ludwik Rydygier Collegium Medicum in Bydgoszcz NCU, Poland
[2] Department of Hygiene, Epidemiology and Ergonomics, Division of Ergonomics and Exercise Physiology, Ludwik Rydygier Collegium Medicum in Bydgoszcz NCU, Poland

Corresponding author:
Natalia Ukleja-Sokołowska
ul. Ujejskiego 75
85-168 Bydgoszcz
E-mail: ukleja@10g.pl

Introduction

Allergic reactions to eggplant (aubergine, *Solanum melongena*) have been rarely reported and are mainly attributed to cross-reactivity with proteins of other fruit and vegetables.[1]

It is widely known that the frequency of allergy to different types of allergens depends on a number of factors, including exposure to a given allergen, socioeconomic factors, comorbidities, the climate zone, and dietary habits.[2,3] The currently described patient is probably one of the first in Poland who has experienced anaphylactic reaction due to eggplant allergy.

The prevalence of IgE-mediated eggplant allergy is estimated at approximately 0.8%, with higher rates of sensitization in females.[4] Eggplant is generally eaten after cooking or baking.[5] Eggplant contains 48 proteins that con potentially be a source of allergic reaction, most of which are not yet fully described.[6] One of the most interesting known eggplant allergens is LTP (lipid transfer protein), which is potentially cross-reactive with allergens of other fruit and vegetables. Other allergens include a protein with weak homology to 7S vicilins,[7] lipoxygenase, protein proteinase inhibitor and 15kDa and 35 kDa heat stable proteins with unknown functions.[8]

In 2009, a very interesting work was published by Babu et al., in which the authors investigated sensitization to different parts of the eggplant fruit, and detected the allergens. It was noticed that most allergens were localized in the eggplant peel (9 allergens; 26–71 kDa range) rather than the pulp (3 allergens; 52–71 kDa); among these, the allergens with molecular weights of 26, 28, 36, and 71 kDa seemed to be heat-stable, and the authors detected the allergens with molecular weights of 43, 45, 64, and 71 kDa to be glycoproteins.[5]

Lipid transfer proteins (LTPs) are plant panallergens, which are described as pure food allergens, and are capable of sensitizing through the gastrointestinal tract. LTPs have been found in many plant-related foods, particularly in those belonging to the *Rosaceae* family, for example peach (Pru p 3) and apple (Mal d 3), but also hazelnut (Cor a 8) from the *Betulaceae* family, walnut (Jug r 3) from the *Juglandaceae* family, peanut (a legume crop, from the *Fabaceae* family), sesame (*Pedaliaceae* family), and celery (*Apiaceae* family).[9,10]

nsLTPs (non-specific LTPs) are mostly present in the peel of fruits, rather than the pulp.[11,12] Interestingly, symptoms after

the consumption of LTP-containing foods can differ. In 2006, Sancho et al. showed that nsLTP levels in apple are greatly dependent on maturity, storage conditions and cultivar.[13]

LTP syndrome is a relatively new name for a clinical pattern of symptoms and natural course of allergy to this panallergen. We now know that there are different recognition patterns of LTPs – some patients only react to peach and apple (*Rosaceae* fruit, where sensitization to peach LTP Pru p 3 can be a marker), while others react to a wide range of foods, including hazelnut or walnut (were mugwort Art v 3 can be a marker).[14,15] Primary sensitization may result from exposure through the oral route, although skin contact may also lead to allergic reactions. This type of allergy is common in the Mediterranean, but not unheard of in other parts of the world. Peach is a typical primary sensitizer.[10] The symptoms may vary from mild oral allergy syndrome (OAS) to life-threatening anaphylactic reaction, even in a single patient.[9,11] The concentration of LTP differs in fruits and vegetables and increases with storage conditions and in certain cultivars.[13]

A case study

Here, we present the case of a 27-year-old male admitted to the hospital with recurrent anaphylactic reactions of unknown cause.

The first allergic reaction appeared in summer 2012; the patient ate a seafood salad with a mixture of vegetables and then went for a fast walk, during which time he experienced swelling of the nose, cheeks, eyelids and tongue, tearing, rhinorrhea and tachycardia. The symptoms retreated after the administration of steroids and antihistamines.

On 31st October 2013, the patient ate fried chicken in a mixture of spices, with a tomato and cucumber salad. Immediately after ingestion he suffered an incident of dyspnea, with swelling of the face and severe pruritic urticaria on his back and upper limbs. In the emergency room, he received steroids and antihistamines with good clinical effect.

On 14th October 2014, the patient ate a homemade dish with pork and eggplant, and drank one beer. Initially, he experienced shortness of breath, and swelling of the face, lips and tongue, which was followed by hypotension, tachycardia of 140/min and a sudden loss of consciousness. He was given epinephrine, crystalloid fluids, steroids and antihistamines, was hospitalized and then discharged with a recommendation to use adrenaline in an auto-injector in the case of anaphylactic reaction.

During the diagnostic pathway, skin prick tests (SPTs) were performed with common inhalant and food allergens (Allergopharma, Nexter); also, prick by prick skin tests were undertaken with selected foods (raw and cocked eggplant pulp and peel, shrimp, banana, scallops, crab, beer, lettuce, tomato, cucumber, chicken meat, pork). The concentrations of IgE against selected food allergens was measured using the ImmunoCap system and the levels of IgE specific to allergen components was established using ImmunoCap ISAC; a value >=0.35 kU/l was considered positive, in accordance with practice commonly accepted in other studies.[16]

The patient had a positive (interpretation according to The Global Allergy and Asthma European Network (GA^2LEN))[17] SPT to allergens of grass and cat dander, but negative results for birch, alder, hazel, weeds, dust mites, molds, and dog dander. Food SPTs were positive to peach, but negative to apple, orange, strawberry, banana, carrot, tomato, celery, potato, hazelnut, peanut, wheat flour, rye flour, rice, soybean, egg, cod, cocoa, and pork.

Results of the prick by prick tests with fresh and cooked eggplant were positive - raw eggplant peel 30/30 mm, pulp 10/10 mm, cooked eggplant peel 7/8 mm, pulp 4/4mm (histamine 4/4mm, control 0/0 mm) and negative with other tested native allergens. Specific IgE concentration was elevated against grass mix (Orchard-, Meadow Fescue, Perennial Rye-, Timothy-, Bluegrass) - 1.63 IU/ml and cat - 0.96 IU/ml. In Poland, it is not possible to estimate IgE to eggplant due to a lack of allergen extract.

The results of ImmunoCap ISAC are displayed in **Table 1**. In ImmunoCap ISAC, there are no allergen components of eggplant available.

Table 1. ImmunoCap ISAC results, only positive results acknowledged

Allergen component	Level of asIgE (ISU-E)	Allergen source
rPhl p 1	1.7	Timothy
rCan f 5	1.2	Dog
Fel d 1	9.6	Cat
nJug r 3	0.5	Walnut (LTP)
rPru p 3	0.6	Peach (LTP)
rPla a 3	1.3	Plane-tree (LTP)

Negative results to following allergen components: nGal d 1, nGal d 2, nGal d 3, nGal d 5, nBos d 4, nBos d 5, nBos d 6, nBos d 8, nBos d lactoferrin, rGad c 1, nPen m 1, nPen m 2, nPen m 4, rAna o 2, rBer e 1, rCor a 1.0401, rCor a 8, nCor a 9, nJug r 1, nJug r 2, nSes i 1, rAra h 1, rAra h 2, rAra h 3, nAra h 6, rAra h 8, rAra h 9, rGly m 4, nGly m 5, nGly m 6, nFag e 2, rTri a 14, rTri a 19.0101, nTri a aA_TI, nAct d 1, nAct d 2, nAct d 5, rAct d 8, rApi g 1, rMal d 1, rPru p 1, nCyn d 1, rPhl p 2, nPhl p 4, rPhl p 5, rPhl p 6, rPhl p 7, rPhl p 11, rPhl p 12, rAln g 1, rBet v 1, rBet v 2, rBet v 4, rCor a 1.0101, nCry j 1, nCup a 1, nOle e 1, nOle e 7, rOle e 9, rPla a 1, nPla a 2, nAmb a 1, nArt v 1, nArt v 3, rChe a 1, rMer a 1, rPar j 2, rPla l 1, nSal k 1, rCan f 1, rCan f 2, nCan f 3, rEqu c 1, nEqu c 3, nFel d 2, rFel d 4, nMus m 1, rAlt a 1, rAlt a 6, rAsp f 1, rAsp f 3, rAsp f 6, rCla h 8, rBlo t 5, nDer f 1, rDer f 2, nDer p 1, rDer p 2, rDer p 10, rLep d 2, rBla g 1, rBla g 2, rBla g 5, nBla g 7, rApi m 1, nApi m 4, rPol d 5, rVes v 5, rAni s 1, rAni s 3, rHev b 1, rHev b 3, rHev b 5, rHev b 6.01, rHev b 8, nMUXF3

Discussion

Based on the medical interview and the results of *in vitro* and *in vivo* tests, we established that the patient is strongly allergic to eggplant, grass and cat dander, although it does not explain all of the symptoms. The fourth anaphylactic reaction was clear, with anaphylaxis due to eggplant consumption. All other reactions were related to different kinds of food, in particularl vegetables.

The clinical pattern of the disease and low, but still present levels of asIgE (antigen specific immunoglobulin E) directed against LTPs from different plant sources may suggest that this patient suffers from LTP syndrome. It is worth emphasizing the features of this case typical for LTP syndrome – unstable clinical course, role of co-factors (exercise, alcohol) and recurrent symptoms after the consumption of fruits and vegetables from

different sources.

There may be several reasons for low levels of LTPs in our patient. An important factor is the time between exposure to LTPs and an examination of the patients' blood serum. In 2011, Asero et al. found that specific IgE levels are only partially predictive of clinical allergy in LTP allergic patients.[18]

Similar results were published in 2012 by Pascal et al., who also found that there was no correlation between LTP-specific IgE levels and the severity of symptoms. In their research, the main offending foods reported by LTP allergic patients and confirmed by SPTs were peach, lettuce, walnut, hazelnut, peanut and green bean. In 40% of patients, cofactors were necessary to induce symptoms.[19] In the present case, cofactors (exercise and alcohol) played an important role in the development of anaphylaxis.

The pathogenic mechanism of cofactors in food allergy is still unclear. An interesting theory suggests that changes in mucosal permeability induced by exercise, medication or other cofactors can enhance allergen absorption through the mucous membranes, and, as a result, enhances exposure to allergens.[20]

Cases of cross allergy between eggplants and latex allergens were described.[21] In the case of our patient, there were no symptoms related to latex and skin prick tests with latex allergen extracts were negative.

The management of patients allergic to LTP is very important, due to large number of anaphylactic reactions in patients with this type of allergy. In 2014, it was emphasized that in the apple allergic population, LTP is most often involved in the occurrence of generalized symptoms in patients.[22] The current patient was advised to carry adrenalin in an auto-injector after his 3rd emergency room visit. It is unfortunately not surprising; there have been researches which state that the majority of patients with anaphylaxis do not receive self-injectable adrenaline to prevent future anaphylactic shock.[23]

Avoidance of sensitizing products is a key part in the management of food allergy. Due to cross-reactivity of nsLTPs, symptoms may appear after the ingestion of fruit and vegetables which previously seemed safe. Patients at risk of anaphylaxis, should carry a rescue set, including adrenaline in an auto-injector. Some patients that are allergic to the LTPs of Rosaceae fruits (around 1/3) may tolerate the pulp of these fruits. Unfortunately, reactions can still be observed when a cofactor is associated. Therefore, as a general recommendation, patients should avoid peeled fruits as well.[19]

In 2007, Asero et al. searched for safe plant-derived foods for LTP-allergic patients. He came to the conclusion that carrot, potato, banana and melon seem safe.[24]

The future for LTP syndrome patients definitely involves a specific immunotherapy developed according to the patient -specific sensitivity profile. There are some interesting cases showing the efficiency of this treatment, but there are no commercially available immunotherapies for these patients.[25]

Knowledge of the clinical pattern of allergy may help patients to understand their disease and prevent life-threatening anaphylactic reactions.

Conclusion

The described patient suffers from a life threatening food allergy. Eggplant allergy, which is very rare in Europe, was confirmed by prick by prick tests. The patient was advised to carry adrenaline in an auto-injector, as well as steroids and antihistamines in case of an anaphylactic reaction.

The clinical pattern of the disease and low, but still present levels of asIgE directed against LTPs from different plant sources, may suggest that this patient suffers from LTP syndrome.

List of abbreviations

Lipid transfer proteins – LTPs
nsLTP – non-specific LTS
Skin prick test – SPT
Oral allergy syndrome - OAS
Antigen specific immunoglobulin E - asIgE
Component resolved diagnosis - CRD

Competing Interests

The authors declare that they have no competing interests.

Financial support

No financial source to declare.

Authors' Contributions

Natalia Ukleja-Sokołowska - interviewed the patient, performed skin prick tests with native allergens, obtained patient permission and drafted the manuscript.

Ewa Gawrońska-Ukleja - designed and coordinated the study, and reviewed the manuscript

Magdalena Żbikowska-Gotz - performed the immunoassays.

Łukasz Sokołowski - reviewed the manuscript, drafted necessary corrections

Zbigniew Bartuzi - reviewed the manuscript, study design and patient diagnosis.

All authors read and approved the final manuscript.

References

1. Pramod SN, Venkatesh YP. Allergy to eggplant (Solanum melongena). J Allergy Clin Immunol. 2004;113:171-3.
2. Tham EH, Lee AJ, Bever HV, Aeroallergen sensitization and allergic disease phenotypes in Asia. Asian Pac J Allergy Immunol. 2016;34:181-9.
3. An SY, Choi HG, Kim SW, Park B, Lee JS, Jang JH, Sung MW, Analysis of various risk factors predisposing subjects to allergic rhinitis. Asian Pac J Allergy Immunol. 2015;33:143-51.
4. Harish Babu BN, Mahesh PA, Venkatesh YP. A cross-sectional study on the prevalence of food allergy to eggplant (Solanum melongena L.) reveals female predominance. Clin Exp Allergy. 2008;38:1795-802.
5. Babu BN, Venkatesh YP. Clinico-Immunological Analysis of Eggplant (Solanum melongena) Allergy Indicates Preponderance of Allergens in the Peel. World Allergy Organ J. 2009;2:192-200.
6. Ramesh KR, Hemalatha R, Vijayendra CA, Arshi UZ, Dushyant SB, Dinesh KB., Transcriptome analysis of Solanum melongena L. (eggplant) fruit to identify putative allergens and their epitopes. Gene. 2016;576:64-71.
7. Jain A, Salunke DM. Purification, identification and preliminary crystallographic studies of an allergenic protein from Solanum melongena. Acta Crystallogr F Struct Biol Commun. 2015;71:221-5.

8. Hoseini-Alfatemi SM, Bayry J, Sharifi-Rad J. IgE response to two new allergen proteins of Solanum melongena L. (eggplant). Immunol Lett. 2015;168:268-70.
9. Pastorello EA, Robino AM. Clinical role of lipid transfer proteins in food allergy. Mol Nutr Food Res. 2004;48:356-62.
10. Asero R. In patients with LTP syndrome food-specific IgE show a predictable hierarchical order. Eur Ann Allergy Clin Immunol. 2014;46: 142-6.
11. Zuidmeer L, van Ree R. Lipid transfer protein allergy: primary food allergy or pollen/food syndrome in some cases. Curr Opin Allergy Clin Immunol. 2007;7:269-73.
12. Marzban G, Puehringer H, Dey R, Brynda S, Ma Y, Martinelli A, et al. Localization and distribution of the major allergens in apple fruits. Plant Sci. 2005;169:387–94.
13. Sancho AI, Foxall R, Rigby NM, Browne T, Zuidmeer L, van Ree R, et al. Maturity and storage influence on the apple (Malus domestica) allergen Mal d 3, a nonspecific lipid transfer protein. J Agric Food Chem. 2006;54: 5098-104.
14. García-Sellés FJ, Díaz-Perales A, Sánchez-Monge R, Alcántara M, Lombardero M, Barber D, et al. Patterns of reactivity to lipid transfer proteins of plant foods and Artemisia pollen: an in vivo study. Int Arch Allergy Immunol. 2002:128:115–22.
15. Fernández-Rivas M, Benito C, González-Mancebo E, de Durana DA, Allergies to fruits and vegetables. Pediatr Allergy Immunol. 2008;19: 675-81.
16. Ukleja-Sokolowska N, Gawronska-Ukleja E, Zbikowska-Gotz M, Socha E, Lis K, Sokołowski L, et al. Analysis of feline and canine allergen components in patients sensitized to pets. Allergy Asthma Clin Immunol. 2016;12:61.
17. Heinzerling L, Mari A, Bergmann KC, Bresciani M, Burbach G, Darsow U, et al. The skin prick test - European standards. Clin Transl Allergy. 2013;3:3.
18. Asero R, Arena A, Cecchi L, Conte ME, Crivellaro M, Emiliani F, et al. Are IgE levels to foods other than rosaceae predictive of allergy in lipid transfer protein-hypersensitive patients? Int Arch Allergy Immunol. 2011; 155:149–54.
19. Pascal M, Muñoz-Cano R, Reina Z, Palacín A, Vilella R, Picado C, et al. Lipid transfer protein syndrome: clinical pattern, cofactor effect and profile of molecular sensitization to plant-foods and pollens. Clin Exp Allergy. 2012;42:1529-39.
20. Lambert GP, Boylan M, Laventure JP, Bull A, Lanspa S. Effect of aspirin and ibuprofen on GI permeability during exercise. Int J Sports Med. 2007;28:722–6.
21. Lee J, Cho YS, Park SY, Lee CK, Yoo B, Moon HB, et al. Eggplant anaphylaxis in a patient with latex allergy. J Allergy Clin Immunol. 2004;113:995-6.
22. Gomez F, Aranda A, Campo P, Diaz-Perales A, Blanca-Lopez N, Perkins J, et al. High prevalence of lipid transfer protein sensitization in apple allergic patients with systemic symptoms. PLoS One. 2014;9:e107304.
23. Braganza S, Acworth J, Mckinnon D, Paediatric emergency department anaphylaxis: different patterns from adults. Arch Dis Child. 2006;91: 159-63.
24. Asero R, Mistrello G, Roncarolo D, Amato S, Detection of some safe plant-derived foods for LTP-allergic patients. Int Arch Allergy Immunol. 2007;144:57-63.
25. Pereira C, Bartolomé B, Asturias JA, Ibarrola I, Tavares B, Loureiro G, et al. Specific sublingual immunotherapy with peach LTP (Pru p 3). One year treatment: a case report. Cases J. 2009;2:6553.

APJAI
Asian Pacific Journal of
Allergy and Immunology

ORIGINAL ARTICLE

Needle length for epinephrine prefilled syringes in children and adolescents: Is one inch needle appropriate?

Wiparat Manuyakorn,[1] Buntita Bamrungchaowkasem,[1] Nichanan Ruangwattanapaisarn,[2] Wasu Kamchaisatian,[1] Suwat Benjaponpitak[1]

Abstract

Background: Intramuscular epinephrine is the first line drug in the treatment of anaphylaxis. This study was to identify the appropriateness of 1 inch needle length for epinephrine prefilled syringes in children.

Methods: Children aged 1 month to 18 years were enrolled. Skin to muscle depth (STMD) and skin to bone depth (STBD) were measured using an ultrasonography at the mid-anterolateral thigh. A 1 inch needle was considered as being appropriate if the STBD was more than 1 inch and the STMD was less than 1 inch.

Results: Seventy five infants, 75 pre-school aged children, 75 school aged children and 147 adolescent were enrolled: 196 (52.7%) children were male. A 1 inch needle length was appropriate for 61% of the infants, for 88% of the preschool children, for 99% of the school aged children and for 95% of the adolescents. Thigh circumference ≥23 cm, BMI ≥16 kg/m^2 and BW ≥ 6 kg in infants provided the sensitivity of 74%-96% in predicting the appropriateness of 1 inch needle. In preschool group, thigh circumference ≥25 cm, BMI ≥13.5 kg/m^2 and BW ≥ 10 kg provided the sensitivity of 98.5-100% in predicting the appropriateness of 1 inch needle. Thigh circumference ≥ 49 cm in adolescents provided the sensitivity of 75% in predicting that a 1 inch needle was too short.

Conclusion: One inch needle length may not be appropriated for intramuscular injection at thigh in all children. Thigh circumference, BMI and body weight are useful for predictor for using the 1 inch needle.

Keywords: Anaphylaxis, children, epinephrine, needle length, intramuscular

From:
[1] Department of Pediatrics, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand
[2] Department of Radiology, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

Corresponding author:
Wiparat Manuyakorn
Department of Pediatrics, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand
Email: mwiparat@hotmail.com

Introduction

Anaphylaxis is a serious allergic reaction that has a rapid onset and can cause fatality. The prevalence of anaphylaxis is gradually increasing worldwide.[1-3] Guidelines for treatment of anaphylaxis have recommended to inject epinephrine intramuscularly at anterolateral thigh.[4,5] Since it can provide the early onset and higher blood level of epinephrine than injection via subcutaneous or intramuscular at deltoid.[6] In addition, those patients with a history of anaphylaxis are strongly indicated for an adrenaline auto-injector.[4,5] However, epinephrine auto-injectors are unavailable or unaffordable in some developing countries. We have recently demonstrated the stability and the sterility of an epinephrine prefilled syringe for use as an alternative treatment for those patients suffering from anaphylaxis.[7]

Centers for Disease Control and Prevention (CDC) has recommended 1 inch (2.54 cm) needle for all thigh intramuscular injection in infants and 1-1¼ inch (2.54-3.17 cm) needle for all thigh intramuscular injection in children (1-18 years).[8] However, a recent study in children aged 1-6 years have reported the over-penetration in 11% of children and under-penetration in 2% of children if 1 inch needle length was used for intramuscular injection at thigh. These authors recommended ⅞-1 inch needle length for this age group.[9] The current study was to identify the appropriateness of a 1 inch needle length for epinephrine prefilled syringes and the predictors for using a 1 inch needle for epinephrine prefilled syringe in children and adolescents.

Methods

A cross-sectional study was performed during January 2015 - June 2015 in children aged between 1 month to 18 years who visited out-patient department, the day care center at Ramathibodi hospital, Bangkok, Thailand and students from secondary schools. The inclusion criteria were children with normal body weight and height. We excluded children who have chronic disease that resulted in an abnormal growth pattern such as chronic lung disease, chronic kidney disease and cardiovascular disease. Baseline characteristic [gender, age, body weight, length/ height, body mass index (BMI), thigh circumference] were recorded. The STBD and the STMD were measured ultrasonographically with a SonoSite M-Turbo Portable Ultrasound Machine (SonoSite Inc, Washington, USA) by a single radiologist in a supine position at the midpoint between the anterior superior iliac spine and the upper pole of patella on the right thigh.[10] Two measurements were taken at each site, and the mean was calculated (**Figure 1**). The needle length was defined as being appropriate if the needle length was more than the measured STMD and it was less than the measured STBD. The needle was defined as a too long needle if the measured STBD was equal or less than the length of the needle and the needle was defined as a too short needle if the measured STMD equal or greater than the length of the needle: 5/8 inch=1.58 cm, 1 inch =2.54 cm and 1.5 inch = 3.81cm.

The study sample size of 316 children [62 infants (1 month - 2 years), 45 preschool children (>2-5 years), 62 school age children (>5-10 years) and 147 adolescents (>10-18 years)] was calculated from our pilot study on the average STBD and STMD in 40 children: 10 infants, 10 preschool-aged children, 10 school-aged children and 10 adolescents. This study was reviewed and approved by the human rights and ethic committee of Faculty of Medicine Ramathibodi Hospital, Mahidol University. All parents were informed written consent and informed written assent for children aged 7 years or older about the objective of the study.

Statistical analysis

All analyses were performed using STATA statistical software version 14 (Stata corp LP, Texus, USA). Descriptive statistical methods (median, mean and frequency) were used to analyze the demographic data. Analysis of difference between groups were performed with the *chi-squared test*. The continuous variables were compared using *ANOVA* with multiple comparisons. Correlations between the variables and the STBD or the STMD were analyzed with the *Pearson correlation*. Receiver operating characteristic (ROC) curves were constructed for the STBD > 25.4 mm and the STMD < 25.4 mm for each parameter (body weight, length/height, thigh circumference and BMI) to identify the most reliable parameter to predict the appropriateness of a 1 inch needle. Receiver operating characteristic (ROC) curves were also constructed for the STMD ≥ 25.4 cm to identify the most reliable parameter to predict the inappropriateness (too short) of a 1 inch needle.

Results

Seventy five infants, 75 pre-school aged children, 75 school aged children and 147 adolescents were enrolled. Age ranged from 1 month to 18 years, with a mean age of 8 years. There was no significant difference in sex among groups.

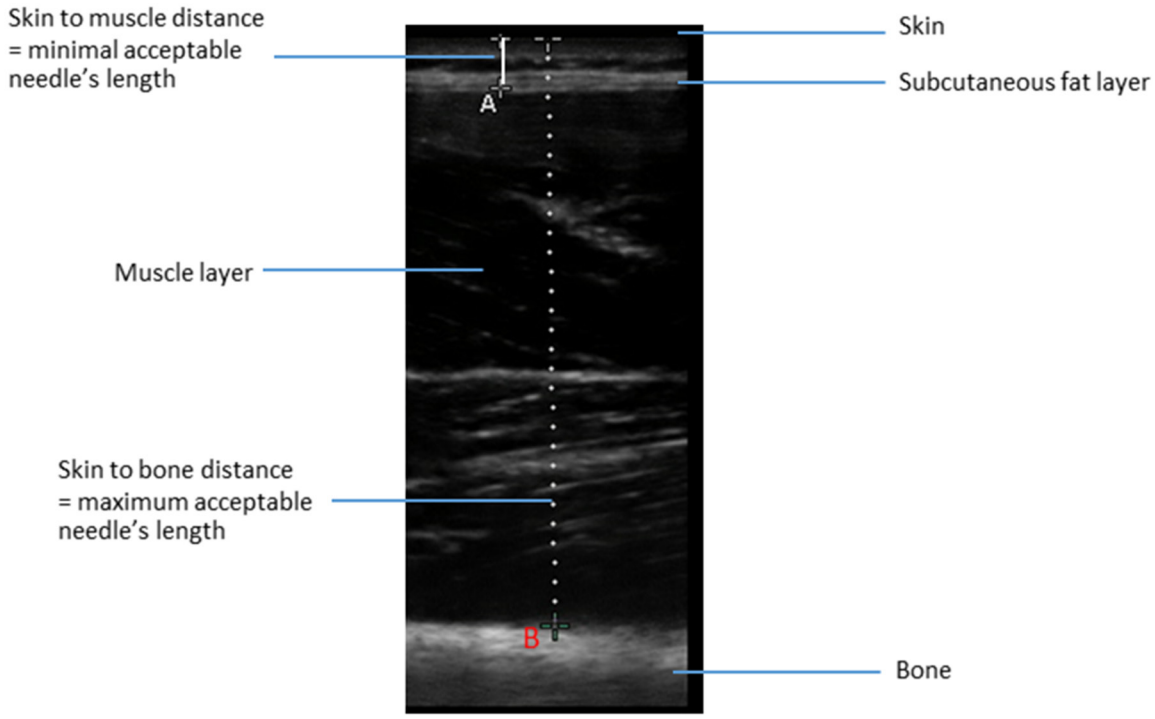


Figure 1. Ultrasound image of anterolateral thigh. A represents skin to muscle depth (STMD), B represents skin to bone depth (STBD).

Correlation of the STBD and the STMD with thigh circumference, body mass index (BMI), body weight, and height

The STBD which can represent the maximum acceptable needle length was very strong correlated with thigh circumference (R=0.90, p<0.001), body weight (R=0.85, p<0.001), and height (R=0.82, p<0.001) and strong correlated with body mass index (BMI) (R=0.70, p<0.001), (**Figure 2**). The STMD which can represent the minimum acceptable needle length was modestly correlated with thigh circumference (R=0.27, p<0.001), body mass index (BMI) (R=0.38, p<0.001), and body weight (R=0.16, p<0.001). In contrast to the STBD, height was not significantly correlated with the STMD (R=0.08, p=0.102) (**Figure 3**).

The appropriateness of a 1 inch needle length among age groups

To find the appropriateness for using of a 1 inch (2.54 cm) needle length for an epinephrine prefilled syringe, number of children who had the STBD equal or less than 2.54 cm (a 1 inch needle is too long) and the STMD equal or greater than 2.54 cm (a 1 inch needle is too short) were calculated. A 1 inch needle length was appropriate for 61.3% of the infants, for 88% of the preschool children, for 98.7% of the school aged children and for 94.6% of the adolescents. It was too long for 38.7% of the infants, for 12% of the preschool children and for 1.3% of the school aged children. However, it was too short for 5.4% of the adolescents (**Table 1**).

Comparison of clinical characteristics between children whom a 1 inch was appropriate and those whom a 1 inch needle was inappropriate

Sex was significantly associated with the appropriateness for a 1 inch needle in infants and adolescents. Sixty nine percent of infants whom 1 inch was not appropriate were male. In contrast, 87.5% of adolescents who were inappropriate for 1 inch needle were female. Infants and preschool children whom 1 inch needle were appropriate had a significantly higher body weight than those children whom 1 inch needle were inappropriate (8.6±1.91 kg *vs* 7.32±2.32 kg, p 0.02 and 13.74± 1.91 kg *vs* 12.06±1.46 kg, p=0.01, respectively). No significant differences in body weight between adolescents in appropriate and inappropriate groups. Adolescents in appropriate group were significantly taller than those in inappropriate group (159.74±9.18 cm *vs* 155.55±3.55 cm, p=0.01). Infants and preschool children in appropriate group had a significantly higher BMI than those children in inappropriate group (16.73±1.53 kg/m^2 *vs* 15.66±1.17 kg/m^2, p 0.002 and 15.74± 1.03 kg/m^2 *vs* 14.44±0.89 kg/m^2, p=0.001, respectively). Infants and preschool children in appropriate group had a significantly

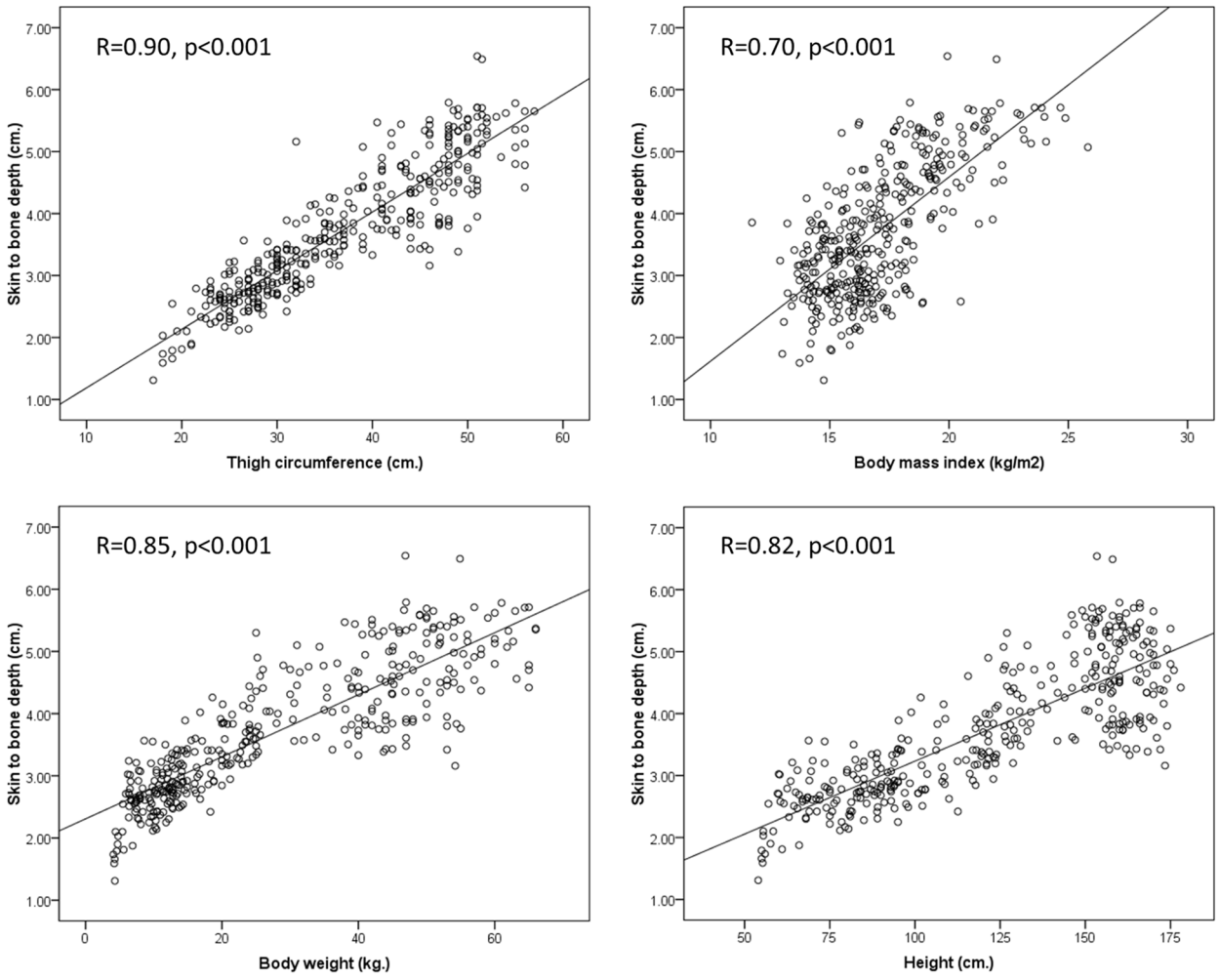


Figure 2. Correlation of skin to bone depth with thigh circumference, body mass index (BMI), body weight, and height.

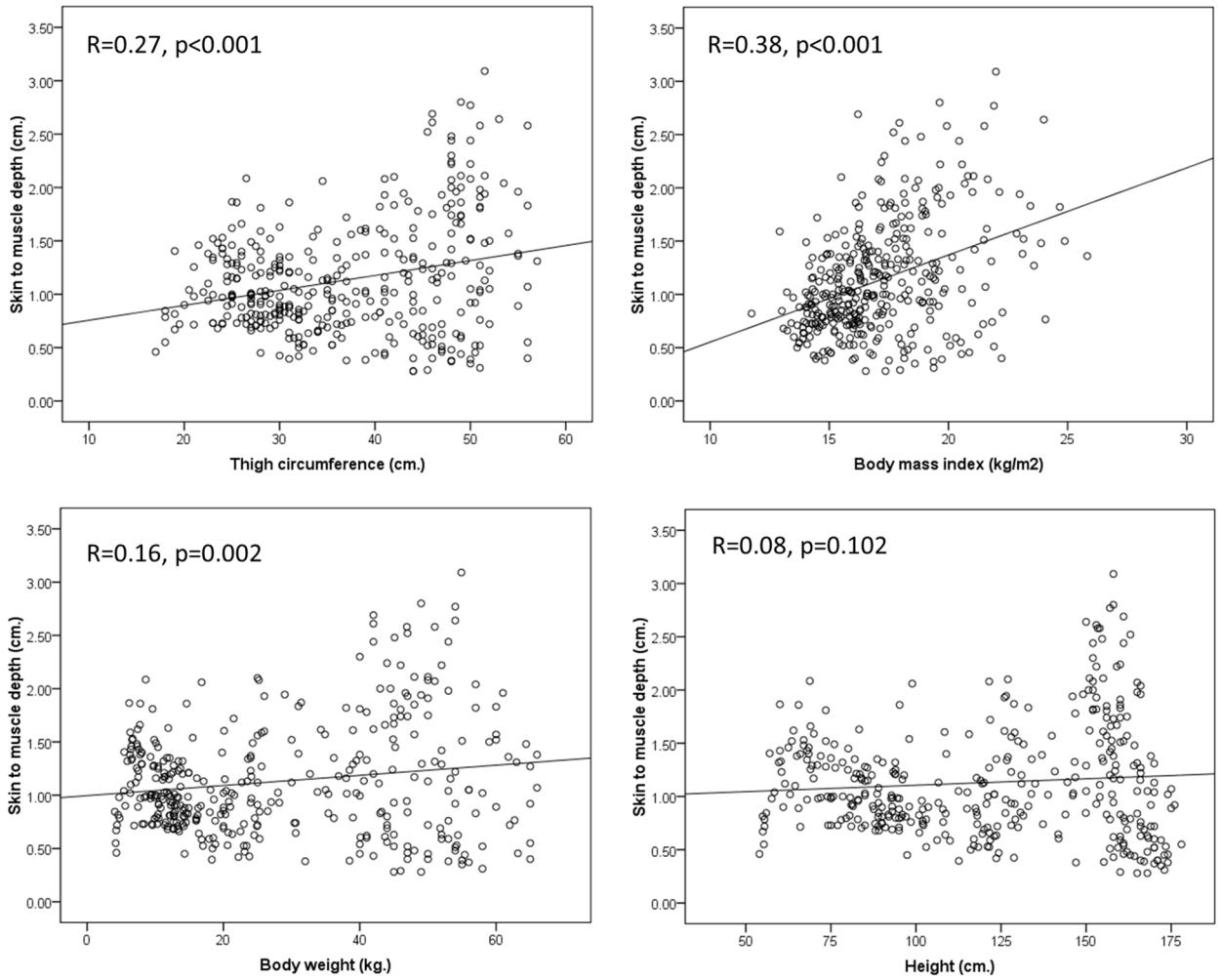


Figure 3. Correlation of skin to muscle depth with thigh circumference, body mass index (BMI), body weight, and height.

Table 1. Comparison of the appropriate rate for 5/8 inch, 1 inch and 1.5 inch needle length among age groups

Age group	Infant (1mo-2year) (n=75)			Preschool (>2 -5 year) (n=75)			School age (>5-10 year) (n=75)			Adolescent (> 10-18 year) (n=147)		
Needle size	1.58 cm (5/8 in)	2.54 cm (1 in)	3.81 cm (1.5 in)	1.58 cm (5/8 in)	2.54 cm (1 in)	3.81 cm (1.5 in)	1.58 cm (5/8 in)	2.54 cm (1 in)	3.81 cm (1.5 in)	1.58 cm (5/8 in)	2.54 cm (1 in)	3.81 cm (1.5 in)
Appropriate n (%)	67 (89.3%)	46 (61.3%)	0 (0%)	72 (96%)	66 (88%)	3 (4%)	63 (84%)	74 (98.7%)	35 (46.7%)	103 (70.1%)	139 (94.6%)	126 (85.7%)
Too short n (%)	7 (9.3%)	0 (0%)	0 (0%)	3 (4%)	0 (0%)	0 (0%)	12 (16%)	0 (0%)	0 (0%)	44 (29.9%)	8 (5.4%)	0 (0%)
Too long n (%)	1 (1.3%)	29 (38.7%)	75 (100%)	0 (0%)	9 (12%)	72 (96%)	0 (0%)	1 (1.3%)	40 (53.3%)	0 (0%)	0 (0%)	21 (14.3%)

longer thigh circumference than those children in inappropriate group. (25.54±2.4 cm *vs* 22.76±3.33 cm, p<0.001 and 29.73± 2.32 cm *vs* 26.06±2.27 cm, p<0.001, respectively). In contrast, adolescents in appropriate group had a significantly lower thigh circumference than adolescents in inappropriate group (46.92±4.74 cm *vs* 50.31±3.39 cm, p=0.05) (**Table 2**).

Comparison between the appropriate rate of a 5/8 inch needle, a 1 inch needle and a 1.5 inch needle length among age group

In infant group, the appropriate rate was at the highest with a 5/8 inch needle (89.7%), followed by a 1 inch needle (61.3%). In preschool children, similar result as infant group was demonstrated. The appropriate rate was at the highest with a 5/8 inch needle (96%), followed by a 1 inch needle (88%). In contrast to infants and preschool children, a 1 inch needle provided the highest appropriate rate in school aged children (98.7%), followed by a 5/8 inch needle (84%) and a 1.5 inch needle (46.7%). The highest appropriate rate in adolescent group was demonstrated in a 1 inch needle (94.6%), followed by a 1.5 inch needle (85.7%) and a 5/8 inch needle (70.1%) (**Table 1**).

Table 2. Comparison in clinical characteristics between group of children whom 1 inch was appropriate and those whom 1 inch needle was inappropriate

groups#	Infants N=75			Pre-school N=75			Adolescent N=147		
appropriate	Yes	No	P -value	Yes	No	P -value	Yes	No	P -value
Age: year, mean (SD)	0.87(0.46)	0.68(0.51)	0.11	2.93(0.72)	2.92(0.62)	0.96	14.46(1.84)	15.2(1.22)	0.26
Gender:N (%)			0.02*			1.00			0.02*
Male	19(41.3)	20(68.97)		28(42.42)	4(44.44)		83(59.7)	1(12.5)	
Female	27(58.7)	9(31.03)		38(57.78)	5(55.56)		56(40.7)	7(87.5)	
Weight: kg, mean (SD)	8.6(1.91)	7.37(2.38)	0.02*	13.74(1.91)	12.06(1.46)	0.01*	48.19(8.64)	49.23(5.22)	0.74
Height: cm mean (SD)	71.4(7.86)	67.51(9.74)	0.06	93.33(6.35)	91.36(6.58)	0.38	159.74(9.18)	155.55(3.55)	0.01*
BMI: kg/m^2, mean (SD)	16.73(1.53)	15.66(1.17)	0.002*	15.74(1.03)	14.44(0.89)	0.001*	18.78(2.39)	20.39(2.49)	0.07
Thigh circumference: cm, mean (SD)	25.54(2.4)	22.76(3.33)	<0.001*	29.73(2.32)	26.06(2.27)	<0.001*	46.92(4.74)	50.31(3.39)	0.05*

#Data of school age children was not included in the table since 1 inch needle was not acceptable in only 1 school aged children,
* Statistical significant

Predictor for the appropriateness of a 1 inch needle for an epinephrine prefilled syringe

Receiver operating characteristic (ROC) curves were constructed for the STBD > 2.54 cm and the STMD < 2.54 cm for each parameter (body weight, length/height, thigh circumference and BMI) to identify parameters for predicting the appropriateness of a 1 inch needle. With a body weight ≥ 6 kg (AUC: 0.72, p=0.001), with a thigh circumference ≥ 23 cm (AUC: 0.73,p=0.001) and with a BMI ≥ 16 kg/m^2 (AUC: 0.64, p=0.05) in infants provided the sensitivity of 74%-96% in predicting the appropriateness for a 1 inch needle. In preschool group, with a body weight ≥ 10 kg (AUC: 0.75, p= 0.02), with a thigh circumference ≥ 25 cm (AUC: 0.87, p< 0.001) and with a BMI ≥ 13.5 kg/m^2 (AUC: 0.83, p=0.002) provided the sensitivity of 99-100% in predicting the appropriateness for a 1 inch needle. In adolescent, ROC curves were constructed for the STMD ≥ 2.54 cm to identify the most reliable parameter to predict the inappropriateness (too short) of a 1 inch needle. With a thigh circumference ≥ 49 cm (AUC: 0.72, p=0.04) provided the sensitivity of 75% in predicting the inappropriateness (too short) of a 1 inch needle (**Table 3**).

Table 3. Cut off value for predicting the appropriateness and the inappropriateness of 1 inch needle

Weight Group	Parameter	AUC	p-value	Sensitivity %, (95% CI)	Specificity %, (95% CI)	Positive predictive value (PPV) %, (95% CI)	Negative predictive value (NPV) %, (95% CI)	Accuracy %, (95% CI)
Prediction for the appropriateness of 1 inch needle (STBD> 2.54 cm and STMD <2.54 cm)								
Infants	Body weight ≥6 kg	0.64	0.05	95.7 (85.2, 99.5)	34.5 (17.9, 54.3)	69.8 (57, 80.8)	83.3 (51.6, 97.9)	72 (60.4, 81.8)
	Thigh circumference ≥23 cm	0.73	0.001	93.5 (82.1, 98.6)	48.3 (29.4, 67.5)	74.1 (61, 84.7)	82.4 (56.6, 96.2)	76 (64.7, 85.1)
	Body mass index ≥16 kg/m^2	0.72	0.001	73.9 (58.9, 85.7)	55.2 (35.7, 73.6)	72.3 (57.4, 84.4)	57.1 (37.2, 75.5)	66.7 (54.8, 77.1)
Pre-school	Body weight ≥10 kg	0.75	0.02	100 (94.6, 100)	11.1 (0.3, 48.2)	89.2 (79.8, 95.2)	100 (2.5, 100)	89.3 (80.1, 95.3)
	Thigh circumference ≥25 cm	0.87	<0.001	100 (94.6, 100)	33.3 (7.5, 70.1)	91.7 (82.7, 96.9)	100 (29.2, 100)	92 (83.4, 97)
	Body mass index ≥13.5 kg/m^2	0.83	0.002	98.5 (91.8, 100)	12.5 (0.3, 52.7)	90.3 (81, 96)	50 (1.3, 98.7)	89.2 (79.8, 95.2)
Prediction for the inappropriateness of 1 inch needle (STMD ≥ 2.54 cm)								
Adolescent	Thigh circumference ≥49 cm	0.72	0.04	75 (34.9, 96.8)	62.6 (54, 70.6)	10.3 (3.9, 21.2)	97.8 (92.1, 99.7)	63.3 (54.9, 71.1)

Discussion

The needle length for delivering epinephrine to intramuscular tissues has previously studied for the suitable of the needle length of epinephrine auto-injectors in adult and children.[11-18] Studies in adult have shown the inadequacy of the epinephrine auto-injector needle length especially in women.[11,13-15] Bhalla et al also have demonstrated that adults whom the needle length of the epinephrine auto-injector were inadequate were shorter, having higher BMI and larger thigh circumference.[13] However, epinephrine auto-injectors are available only in 59.1% of countries according to a recent report from the World Allergy Organization.[19] It is also unaffordable in many developing countries. Epinephrine prefilled syringes can be alternative options for anaphylaxis patients.[20] In addition, the needle length of epinephrine auto-injectors range from 1.17 to 2.5 cm depending on the manufacturer.[12] The majority of the previous studies regarding on the adequacy of the epinephrine auto-injector needle length used the needle length of Epipen® as a reference. Epinephrine auto-injectors also require pressure trigger for the epinephrine delivery which produces the compression pressure on the skin resulting in the shortening the length of the STMD. As a result, studies regarding the needle length for epinephrine auto-injectors may not apply for the needle length for epinephrine prefilled syringe.

We have demonstrated that a 1 inch needle which is the recommended needle length for all thigh intramuscular injection in children was not appropriated for the epinephrine prefilled syringe in a majority of infants and preschool children. A 1 inch needle was appropriate in only 61% of the infants and 88% of the preschool children. It was too long in 39% of the infants and 12% of the preschool children. We found that a 5/8 inch needle was more appropriate in these age groups. Lippert and colleagues has reported that one inch needle length was too long for intramuscular injection at thigh in 11% of children aged 1-6 years and they suggested 7/8-1 inch needle length in children aged less than 6 year.[9] A study in 40 infants and 18 toddlers found subcutaneous fat thickness plus muscle thickness at thigh (equal to the STBD in our study) was less than 25.4 mm (1 inch) in all infants and toddlers which means that a 1 inch needle is too long for intramuscular injection at thigh.[21] We have found that the male infants had a significantly lower percentage for the appropriateness for a 1 inch needle than that was demonstrated in the female infants (48.7% vs 75%, p=0.03). Furthermore, body weight, BMI and thigh circumference were significantly differences between infants and preschool children whom 1 inch needle were appropriate and those children with 1 inch needle inappropriate.

The association of subcutaneous fat thickness and BMI has been shown in studies in adult and children.[11,13,17] We have proposed to use for a thigh circumference ≥ 23 cm, with a BMI ≥16 kg/m^2 and with a body weight ≥ 6 kg for predicting the appropriateness for using 1 inch needle in infants with the sensitivity of 74-96%, the positive predictive value (PPV) 70-74% and negative predictive value (NPV) 57-83%. We also have proposed the cut off value for preschool children: with a thigh circumference ≥ 25 cm, with a BMI ≥ 13.5 kg/m^2 and with a body weight ≥ 10 kg as predictors for the appropriateness for using a 1 inch needle for epinephrine prefilled syringe with the sensitivity of 98-100%, the positive predictive value (PPV) of 89-92% and the negative predictive value (NPV) of 50-100%. We would suggest a 5/8 inch needle for preparing the epinephrine prefilled syringe in children who have one of these parameters less than these specific value for preventing intraosseous injection from the too long needle especially in male infants.

In contrast to infants and preschool children, a 1 inch needle is appropriate for almost all school age children. In addition, a 1 inch needle may be too short for intramuscular injection at thigh in adolescent especially in female. A study in adult have shown the higher STMD in female than male.[15] We have proposed to use for a thigh circumference ≥ 49 cm as a predictor for using a needle length longer than 2.54 cm for preparing the epinephrine prefilled syringe in adolescent with the sensitivity of 75%, the specificity of 63%, the negative predictive value (NPV) of 98%. The limitation of our study is that we have enrolled only children with normal growth parameters. Further studies on the STMD and the STBD in all children and adolescents are needed for the guidance for the needle length of epinephrine prefilled syringes for all children and adolescents.

In conclusion, a 1 inch needle as recommended for intramuscular injection at thigh in children and adolescents may not appropriate for using for the epinephrine prefilled syringe in all children and adolescents. It is too long for infants and preschool children and too short for female adolescents. Thigh circumference, BMI and body weigh are useful parameters for predicting the ability of using a 1 inch needle for an epinephrine prefilled syringe.

Acknowledgement

All authors have no conflict of interest. WM, BB, WK, and SB designed the study and wrote the manuscript. BB and NR contributed to data collection. WM and BB performed the statistical analysis and interpretation the results. All authors read and approved the final manuscripts. The authors would like to thank you Ms Dollapas Punpanich for helping in the statistical analysis. We are grateful to Berli Jucker Public Company for supporting ultrasonogram. This study was supported by a research grant from Faculty of Medicine Ramathibodi hospital, Mahidol University, Bangkok, Thailand.

References

1. Manuyakorn W, Benjaponpitak S, Kamchaisatian W, Vilaiyuk S, Sasisakulporn C, Jotikasthira W. Pediatric anaphylaxis: triggers, clinical features, and treatment in a tertiary-care hospital. Asian Pac J Allergy Immunol. 2015;33:281-8.
2. Tejedor Alonso MA, Moro Moro M, Mugica Garcia MV. Epidemiology of anaphylaxis. Clin Exp Allergy. 2015;45:1027-39.
3. Park HW. Predictors of anaphylaxis in korean adults. Allergy Asthma Immunol Res. 2015;7:1-2.
4. Lieberman P, Nicklas RA, Randolph C, Oppenheimer J, Bernstein D, Bernstein J, et al. Anaphylaxis--a practice parameter update 2015. Ann Allergy Asthma Immunol. 2015;115:341-84.
5. Simons FE, Ebisawa M, Sanchez-Borges M, Thong BY, Worm M, Tanno LK, et al. 2015 update of the evidence base: World Allergy Organization anaphylaxis guidelines. World Allergy Organ J. 2015;8:32.
6. Simons FE, Gu X, Simons KJ. Epinephrine absorption in adults: intramuscular versus subcutaneous injection. J Allergy Clin Immunol. 2001;108:871-3.

7. Kerddonfak S, Manuyakorn W, Kamchaisatian W, Sasisakulporn C, Teawsomboonkit W, Benjaponpitak S. The stability and sterility of epinephrine prefilled syringe. Asian Pac J Allergy Immunol. 2010;28:53-7.
8. Vaccine administration guidelines. In: Atkinson WWS, Hamborsky J, editors. 13th ed. Centers for Disease Control and Prevention. Epidemiology and prevention of vaccine-preventable diseases. Washington D.C. Public Health Foundation; 2015; p79-106.
9. Lippert WC, Wall EJ. Optimal intramuscular needle-penetration depth. Pediatrics. 2008;122:e556-63.
10. Manuyakorn W, Bamrungchaowkasem B, Ruangwattanapaisarn N, Kamchaisatian W, Benjaponpitak S. Optimal needle length for epinephrine prefilled syringe in children. Ann Allergy Asthma Immunol. 2017;118: 740-1 e1.
11. Johnstone J, Hobbins S, Parekh D, O'hickey S. Excess subcutaneous tissue may preclude intramuscular delivery when using adrenaline autoinjectors in patients with anaphylaxis. Allergy. 2015;70:703-6.
12. Song TT, Lieberman P. Epinephrine auto-injector needle length: what is the ideal length? Curr Opin Allergy Clin Immunol. 2016;16:361-5.
13. Bhalla MC, Gable BD, Frey JA, Reichenbach MR,Wilber ST. Predictors of epinephrine autoinjector needle length inadequacy. Am J Emerg Med. 2013;31:1671-6.
14. Tsai G, Kim L, Nevis IF, Dominic A, Potts R, Chiu J, et al. Auto-injector needle length may be inadequate to deliver epinephrine intramuscularly in women with confirmed food allergy. Allergy Asthma Clin Immunol. 2014;10:39.
15. Song TT, Nelson MR, Chang JH, Engler RJ, Chowdhury BA. Adequacy of the epinephrine autoinjector needle length in delivering epinephrine to the intramuscular tissues. Ann Allergy Asthma Immunol. 2005;94:539-42.
16. Dreborg S, Wen X, Kim L, Tsai G, Nevis I, Potts R, et al. Do epinephrine auto-injectors have an unsuitable needle length in children and adolescents at risk for anaphylaxis from food allergy? Allergy Asthma Clin Immunol. 2016;12:11.
17. Stecher D, Bulloch B, Sales J, Schaefer C, Keahey L. Epinephrine auto -injectors: is needle length adequate for delivery of epinephrine intramuscularly? Pediatrics. 2009;124:65-70.
18. Kim L, Nevis IF, Tsai G, Dominic A, Potts R, Chiu J, et al. Children under 15 kg with food allergy may be at risk of having epinephrine auto-injectors administered into bone. Allergy Asthma Clin Immunol. 2014;10:40.
19. Simons FE. World Allergy O. Epinephrine auto-injectors: first-aid treatment still out of reach for many at risk of anaphylaxis in the community. Ann Allergy Asthma Immunol. 2009;102:403-9.
20. Pepper AN, Westermann-Clark E, Lockey RF. The High Cost of Epinephrine Autoinjectors and Possible Alternatives. J Allergy Clin Immunol Pract. Forthcoming 2017.
21. Groswasser J, Kahn A, Bouche B, Hanquinet S, Perlmuter N, Hessel L. Needle length and injection technique for efficient intramuscular vaccine delivery in infants and children evaluated through an ultrasonographic determination of subcutaneous and muscle layer thickness. Pediatrics. 1997;100:400-3.

ORIGINAL ARTICLE

Asian Pacific Journal of
Allergy and Immunology

APJAI

Performance evaluation of the Bioneer *AccuPower*® HIV-1 Quantitative RT-PCR kit: Comparison with the Roche COBAS® AmpliPrep/COBAS TaqMan® HIV-1 Test Ver.2.0 for quantification of HIV-1 viral load in Indonesia

Agus Susanto Kosasih,[1] Christine Sugiarto,[1] Hubertus Hosti Hayuanta, Runingsih Juhaendi,[1] Lyana Setiawan[1]

Abstract

Background: Measurement of viral load in human immunodeficiency virus type 1 (HIV-1) infected patients is essential for the establishment of a therapeutic strategy. Several assays based on qPCR are available for the measurement of viral load; they differ in sample volume, technology applied, target gene, sensitivity and dynamic range. The Bioneer *AccuPower*® HIV-1 Quantitative RT-PCR is a novel commercial kit that has not been evaluated for its performance.

Objective: This study aimed to evaluate the performance of the Bioneer *AccuPower*® HIV-1 Quantitative RT-PCR kit.

Methods: In total, 288 EDTA plasma samples from the Dharmais Cancer Hospital were analyzed with the Bioneer *AccuPower*® HIV-1 Quantitative RT-PCR kit and the Roche COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 version 2.0 (CAP/CTM v2.0). The performance of the Bioneer assay was then evaluated against the Roche CAP/CTM v2.0.

Results: Overall, there was good agreement between the two assays. The Bioneer assay showed significant linear correlation with CAP/CTM v2.0 (R2=0.963, p<0.001) for all samples (N=118) which were quantified by both assays, with high agreement (94.9%, 112/118) according to the Bland-Altman model. The mean difference between the quantitative values measured by Bioneer assay and CAP/CTM v2.0 was 0.11 Log10 IU/mL (SD=0.26).

Conclusion: Based on these results, the Bioneer assay can be used to quantify HIV-1 RNA in clinical laboratories.

Keywords: HIV-1, PCR, viral load, *AccuPower*®, COBAS® AmpliPrep/COBAS® TaqMan®

From:
[1] Clinical Pathology Laboratory, Dharmais Cancer Hospital, West Jakarta, Indonesia

Corresponding author:
Agus Susanto Kosasih
Email: agus.kosasih0102@gmail.com

Introduction

HIV is the responsible etiologic agent for the onset of AIDS, a disease characterized by the depletion of CD4+ helper T-cells.[1] AIDS leaves the host susceptible to opportunistic infections and cancer development, which frequently result in mortality.[2] At the end of 2013, there were 35 million people living with HIV. This number has risen as more people are living longer because of antiretroviral therapy (ART), alongside the number of new HIV infections which, although declining, is still very high.

Measurement of viral load in HIV-1 infected patients is essential for the establishment of a therapeutic strategy. In 2013, the World Health Organization recommended that, with the exception of dried blood spot samples, the threshold for detection of virological failure should be lowered to 1,000 copies/mL.[3] Thus, the commercial HIV-1 quantitative assay should be able to detect and quantify at least 1,000 copies/mL. Several commercial kits utilizing different principles, such as nucleic acid sequence based amplification (NASBA), branched DNA (bDNA) and reverse transcriptase real-time PCR (RT-qPCR) have been developed to detect and quantify HIV-1 RNA.[4] These kits are able to detect and quantify HIV-1 viral load of 1,000 copies/mL, which is within the linear range of viral load assay claimed by manufacturers.[5]

Indonesia, a country with a population of 249.9 million in 2013, has an estimated HIV prevalence of 0.5% among the 15 to 49-year-old age group. The situation in Indonesia is a cause for concern, as new HIV infections have increased by

48% from 2005 to 2013, and the country's share of new HIV infections in the Asian/the Pacific region reached 23% in 2013, second only to India.[6] The high incidence of HIV-1 infection in Indonesia consequently translates into a high demand for HIV-1 viral load measurement. Unfortunately, HIV-1 viral load measurement is still considered expensive, and requires expensive analyzers and skilled operators, and is therefore still underutilized.

The Bioneer *AccuPower*® HIV-1 Quantitative RT-PCR kit (Bioneer, Daejeon, Korea) is a novel commercial kit which applies TaqMan technology and targets the HIV-1 *pol* (integrase) gene for the quantification of HIV-1 group M, N and O in human EDTA-treated plasma. The dynamic range of *AccuPower*® HIV-1 Quantitative RT-PCR kit is 100~100,000,000 IU/mL. This system uses only 0.4 mL plasma, and has a short turnaround time of 3.5 hours. Thus, the Bioneer kit and *ExiStation*™ Universal Molecular Diagnostic System offer an affordable alternative for HIV-1 viral load testing for developing countries, but its performance has not been evaluated in Indonesia.

In this study, the performance of the Bioneer *AccuPower*® HIV-1 Quantitative RT-PCR kit was evaluated in comparison to the Roche CAP/CTM v2.0 assay, which is the assay currently used at the Dharmais Cancer Hospital, a referral hospital for HIV testing in Indonesia.

Material and Methods

Ethical approval

This study has been approved by the Dharmais Cancer Hospital Ethics Committee (approval number: 060/KEPK/IX/2016).

Clinical samples and WHO panel

For the determination of the limit of detection (LoD), a WHO panel (WHO International Standard Third HIV-1 International Standard, NIBSC code 10/152, subtype B) was used. For the evaluation of HIV-1 subtype detection, the Second WHO International Reference Panel Preparation for HIV-1 Subtypes for NAT (Main) (NIBSC code: 12/224) and the First WHO International Reference Preparation for HIV-1 CRFs (NIBSC code: 13/214) were used. The panels included 14 subtypes from Group M, including subtype AE and Circulating Recombinant Form (CRF)01_AE which were the subtypes reported as the most prevalent in some provinces in Indonesia,[7,8] plus Group N and Group O.

A total of 288 EDTA plasma samples from outpatients requesting HIV-1 RNA viral load testing were collected at the Dharmais Cancer Hospital, Indonesia consisting of fresh samples (N=94) which were stored at 4-8°C before testing and frozen samples (N=194) which were stored at -20°C until testing.

Measurement of viral load

Bioneer AccuPower® HIV-1 Quantitative RT-PCR kit

The HIV-1 RNA was quantified using an *ExiStation*™ Universal Molecular Diagnostic System which consisted of three *ExiPrep*™ 16 Dx nucleic acid extraction instruments and one *Exicycler*™ 96 real-time quantitative thermocycler. *ExiStation*™ manager software controlled the entire process of nucleic acid preparation, amplification, automatic data acquisition and analysis, and finally delivered a report to the user. The *ExiPrep*™ 16 Dx automated the extraction and purification of nucleic acids from clinical specimens by utilizing magnetic particle technology. All reagents for extraction were included in the cartridge of the *ExiPrep*™ Dx Viral RNA kit. Extracted RNA was eluted into diagnostic reaction tubes directly, and thus RT-qPCR on the *Exicycler*™ 96 was performed without any manual pipetting steps.[9]

HIV-1 RNA was extracted from 0.4 mL of EDTA plasma using the *ExiPrep*™ Dx Viral RNA kit and the *ExiPrep*™ 16 Dx of the ExiStation™ system. The extracted HIV-1 RNA was automatically loaded into the PCR tubes of the AccuPower® HIV-1 Quantitative RT-PCR kit, and RT-qPCR was performed by the *Exicycler*™ 96 real-time quantitative thermocycler with the following program: 55°C for 15 min; 95°C for 5 min; 45 cycles of 95°C for 5 sec, 55°C for 5 sec and fluorescence scan; 25°C for 1 min. All procedures were conducted according to the manufacturer's instructions. The concentration of HIV-1 RNA was determined based on the threshold cycle (Ct), and the corresponding RNA concentration (IU/mL) was calculated using a standard curve. An internal positive control consisting of RNA sequences unrelated to the HIV-1 target sequence, was preloaded in each sample loading tube from the beginning of RNA extraction from specimens to determine whether the nucleic acids were properly extracted and amplified in each reaction and whether PCR was inhibited by the sample.

Roche Cobas AmpliPrep/Cobas TaqMan HIV-1 test, v2.0 (CAP/CTM v2.0)

The Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 test v2.0 (CAP/CTM v2.0) (Roche Diagnostics Ltd, Burgess Hill, UK) uses a fully automated analyzer for the quantification of HIV-1 groups M and O in human plasma. CAP/CTM v2.0 targets both the *gag* and LTR regions using two dual-labeled hybridization probes with a dynamic range of 34-17,000,000 IU/mL and LoD of 34 IU/mL.[10] HIV-1 RNA was obtained from a 0.85 mL of EDTA plasma using the COBAS AmpliPrep system. RNA was extracted using the COBAS AmpliPrep system prior to being automatically transferred to the COBAS TaqMan system for amplification and detection. All procedures were conducted according to the manufacturer's instructions.

Statistical analysis

The correlation coefficient (r) was used to assess the strength of the linear association between the $\log_{10}$ IU/mL levels in the quantified samples measured by the two assays. The Bland-Altman method was used to assess the level of agreement between the paired measurements.[11] The correlation coefficient, the Bland-Altman plot, mean differences and standard deviation (SD) were generated using SPSS 18.0 software.

Results

Limit of detection

The LoD of the Bioneer HIV-1 assay was determined by analyzing a dilution series of the WHO Panel (Third HIV-1 International Standard, subtype B) at concentrations of 100,

50, 25, 12.5, 6.3, and 3.1 IU/mL. The detection rates of each concentration are summarized in **Table 1**. The proportion of positive results from each concentration was analyzed to calculate the 95% hit detection limit, which was 38.0 IU/mL.

Table 1. Limit of Detection of the Bioneer HIV-1 test using the WHO International Panel.

Concentration (IU/mL)	Percent detected (Number detected / Number tested)
100	100 (30/30)
50	100 (31/31)
25	93.5 (29/31)
12.5	56.3 (18/32)
6.3	32.3 (10/31)
2.1	29 (9/31)

The detection of HIV-1 subtypes by the assay was evaluated using the WHO subtype panel, which includes 14 subypes: A, C, D, AE, F, G, AG-GH, CRF_11AJ, CRF_02AG, CRF_01AE, CRF_01AGJU, CRF_24BG, J, and CRF_ADG. The assay detected all 14 subtypes at a concentration of 100, and at 50 IU/mL when four replicates of each concentration were tested. The assay detected positive all of four replicates of Group N at a level of 50 IU/mL and Group O at a level of 100 IU/mL.

Performance of the AccuPower® HIV-1 Quantitative RT-PCR kit versus CAP/CTM v2.0

Of the 288 EDTA plasma samples, HIV-1 was not detected in 107 samples, detected below the lower limit of quantification (LLoQ) in 16 samples, and quantified in 118 samples by both assays (**Table 2**). Among the 118 quantified samples, 17 samples were quantified between the LLoQ and 1,000 copies/mL. The average viral loads (log_{10} IU/mL; the conversion factor for HIV RNA was 1.74 copies/IU) in the 17 samples measured by the Bioneer assay and CAP/CTM v2.0 were 2.44 (SD=0.27) and 2.53 (SD=0.36), respectively.

Table 2. Comparison of HIV-1 viral load determinations by both assays.

		No. of samples with the following result with CAP/CTM v2.0			
		Undetected	<LloQ	Quantified	Total
Results with Bioneer assay	Undetected	107	14	4	125
	<LLoQ[a]	5	16	19	40
	Quantified	4	1	118	123
	Total	116	31	141	288

[a] LLoQ: Lower limit of quantification

The Bioneer assay showed a significant linear correlation with the CAP/CTM v2.0 assay (R^2=0.963, p<0.001) for all samples (N=118) that were quantified by both assays (**Figure 1A**), with high agreement (94.9%, 112/118) according to the Bland-Altman model (**Figure 1B**). The fitted regression equation was: Bioneer assay = 1.0563×CAP/CTM v2.0 - 0.1549. The mean difference between the quantitative values measured by the Bioneer assay and the CAP/CTM v2.0 assay was 0.11 log_{10} IU/mL (SD=0.26).

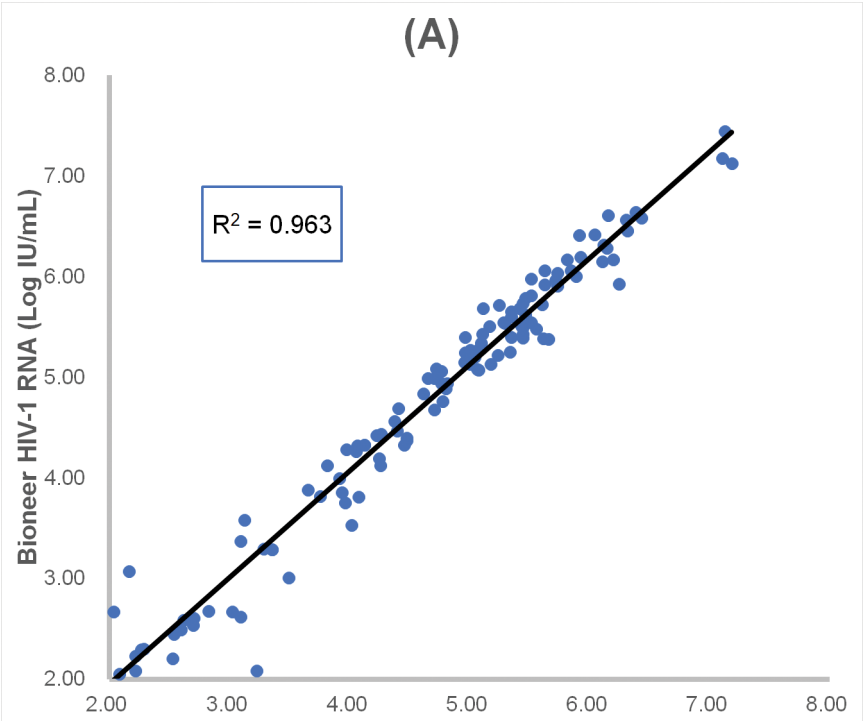


Figure 1. Correlation between HIV-1 RNA levels from all quantified samples obtained using the Bioneer assay and the CAP/CTM v2.0, R^2 = 0.963 (A).

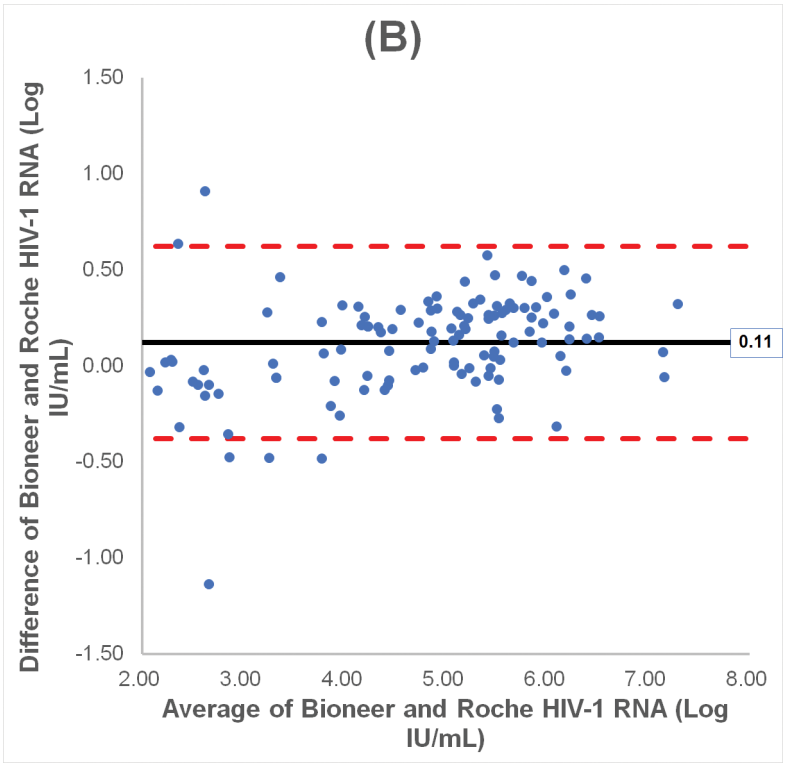


Figure 1. (Continued) Agreement between the Bioneer assay and CAP/CTM v2.0 calculated using the Bland–Altman model (B). Solid horizontal lines indicate the mean values, and red-dashed horizontal lines indicate the ± 2 SD values.

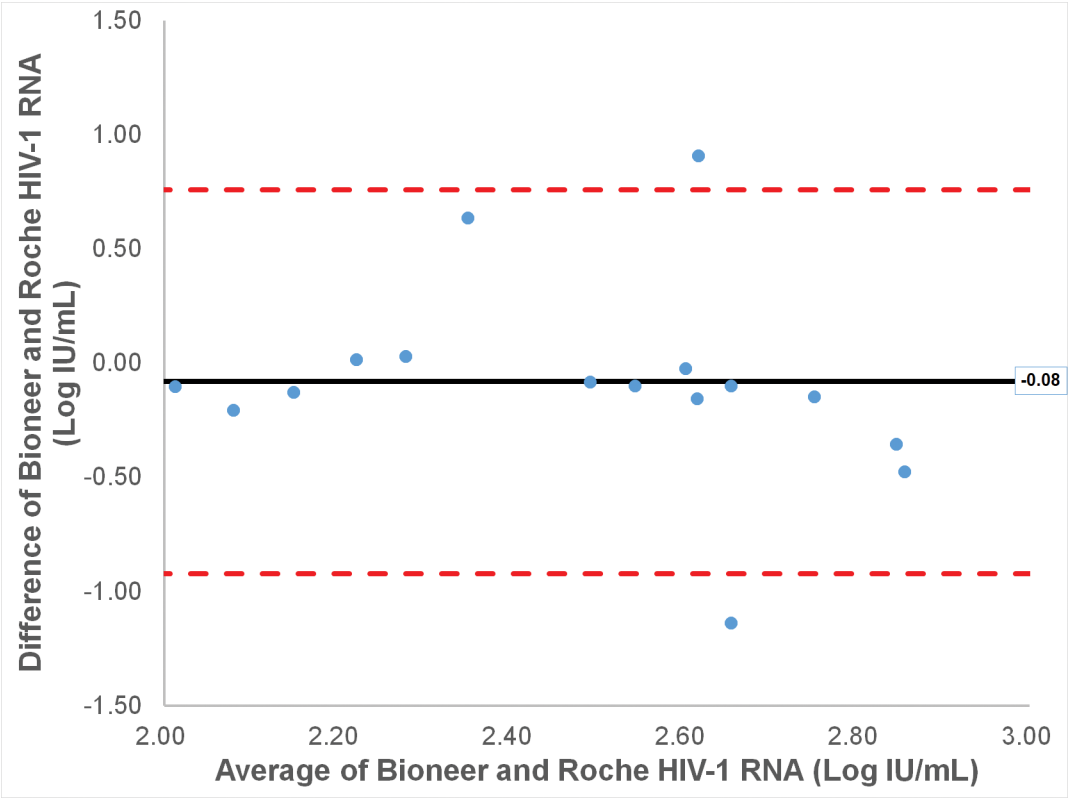


Figure 2. Bland-Altman model of 17 low copy samples with VL between LLoQ and 1,000 copies/mL. Solid horizontal line indicates the mean value, and red-dashed horizontal lines indicate the ± 2 SD values.

The Bland-Altman model of the 17 low copy samples which were quantified between the LLoQ and 1,000 copies/mL showed good agreement between the two assays (88.2%, 15/17). A difference of >0.3 $\log_{10}$ IU/mL was observed in two samples (11.8%), in which the concentrations ($\log_{10}$ IU/mL) were 2.62 and 2.67 by Bioneer (3.09 and 3.02 by CAP/CTM v2.0, respectively); a difference of >0.5 $\log_{10}$ IU/mL was observed in three samples (17.6%), in which the concentrations ($\log_{10}$ IU/mL) were 2.09, 3.07, and 2.67 by Bioneer (3.22, 2.16, and 2.03 by CAP/CTM v2.0, respectively). The mean difference in 17 low copy samples between the quantitative values measured by the Bioneer assay and the CAP/CTM v2.0 assay was -0.08 $\log_{10}$ IU/mL (SD=0.43) (**Figure 2**). Overall, the quantification of low copy samples was similar by both assays.

The average viral loads ($\log_{10}$ IU/mL) in the 118 samples measured by the Bioneer assay and CAP/CTM v2.0 were 4.81 (SD=1.31) and 4.70 (SD=1.21), respectively. There were four samples that were quantified by CAP/CTM v2.0, but not detected by the Bioneer assay. The viral loads were 57, 86, 118, and 130 IU/mL by CAP/CTM v2.0. In contrast, four samples were quantified by the Bioneer assay, but not detected by CAP/CTM v2.0. The viral loads determined by the Bioneer assay were 584, 404, 135, and 204 IU/mL.

Discussion

HIV-1 viral load is the most important indicator of initial and sustained response to ART and should be measured in all HIV-infected patients at entry into care, at the initiation of therapy, and on a regular basis thereafter.[12] Current international guidelines and clinical trials define virological suppression as the achievement and maintenance of an HIV-1 RNA level of 50 copies/mL or to levels below assay limits as the virologic endpoint of successful ART. In contrast, the HIV RNA threshold for defining virological failure differs between guidelines. The WHO guidelines define virological failure as a viral load that is persistently above 1,000 copies/mL after ≥6 months on ART.[3] The British HIV Association (BHIVA),[13] International Antiviral Society (IAS),[14] European AIDS Clinical Society (EACS),[15] and Department of Health and Human Services (DHHS) guidelines consider virological failure to be a confirmed viral load of more than 400, 200, 50, or 48 copies/mL after suppression, respectively.[12,16]

In the present study, 288 clinical samples were tested for the evaluation of the Bioneer assay in comparison with Roche CAP/CTM v2.0. Of 288 samples, 118 samples were quantified by the Bioneer assay and CAP/CTM v2.0. All 107 negative samples were negative in both assays, indicating that the Bioneer assay has a good specificity for the test. There were 19 samples that were undetected by one assay and determined below the LLoQ by the other assay. Five samples were detected below the LLoQ in the Bioneer assay while these were not detected by CAP/CTM v2.0, and 14 samples were detected below the LLoQ in CAP/CTM v2.0 but not detected in the Bioneer assay. There were 20 samples below the LLoQ by one assay and quantified by the other assay. Of the 20 samples, one sample was quantified by the Bioneer assay (238 IU/mL) but detected below the LLoQ by CAP/CTM v2.0, and 19 samples were quantified by CAP/CTM v2.0 and detected below the LLoQ by the Bioneer assay. The average HIV-1 RNA concentration of the 19 samples was 109 IU/mL (SD=80.6). These results were likely caused by different LLoQ of each assay. The LLoQ of CAP/CTM v2.0 is 34 IU/mL, whereas the LLoQ of the Bioneer assay is 100 IU/mL.

A total of eight samples were discordant. The Bioneer assay quantified four samples in which the average concentration of HIV-1 RNA was 331 IU/mL, all of which were undetected by the CAP/CTM v2.0 assay. CAP/CTM v2.0 quantified four samples in which the average concentration of HIV-1 RNA was 98 IU/mL, all of which were undetected by the Bioneer assay. This result may be caused by the different primers. In a 2004 case report in Thailand, a seven-year-old child with HIV-1 subtype (CRF)01_AE, which is the most prevalent subtype in Indonesia, had undetectable HIV-1 RNA and DNA using env/pol primers, but was positive using gag/pol.[17] Bioneer uses a pol primer, while CAP/CTM v2.0 uses gag/LTR primers. Confirmatory tests for the discordant results of both assays were not performed due to the lack of samples.

A significant correlation (R^2=0.963, p<0.001) between the two assays was found for the 118 quantified samples, even though the Bioneer assay uses 0.4 mL of plasma while CAP/CTM v2.0 uses 0.85 mL of plasma for the test. Bland-Altman analysis revealed that the differences for 112 samples (94.9%) among the 118 samples were within the 2SD value, while those for the other six samples were outside the 2SD value, thus indicating good agreement between the two assays for the 118 samples assessed as quantified by both assays.

Most guidelines recommend the use of a single quantification assay for therapeutic monitoring of HIV-1 infected patients. Furthermore, several current guidelines advise against switching ART in HIV-1 infected patients when the HIV-1 viral load remains at 200 to 1,000 copies/mL, taking into account the possibility that it may represent a blip.[3,12-15,18] On the other hand, during ART, a careful assessment of persistent HIV-1 RNA in the range of 200 to 1,000 copies/mL is recommended, since viral evolution and drug resistance mutations might accompany low level viral replication, subsequently leading to virological failure.[19-22] The Bioneer assay showed good agreement and significant quantification of the HIV-1 viral load with CAP/CTM v2.0 using low copy samples. These results indicate that the Bioneer assay is able to quantify low copy HIV-1 viral load in HIV-1 infected patients on ART.

Conclusion

In conclusion, a good agreement was observed between the two assays. The Bioneer assay met the performance criteria as an alternative for HIV-1 RNA quantification in clinical laboratories.

Acknowledgements

We thank Bioneer Corp. for providing the AccuPower HIV-1 Quantitative RT-PCR kit for this study.

References

1. O'Brien WA, Hartigan PM, Martin D, Esinhart J, Hill A, Benoit S, et al. Changes in plasma HIV-1 RNA and CD4+ lymphocyte counts and the risk of progression to AIDS. Veterans Affairs Cooperative Study Group on AIDS. N Engl J Med. 1996;334:426-31.

2. Naidoo K, Naidoo K, Padayatchi N, Abdool Karim Q. HIV-associated tuberculosis. Clin Dev Immunol. 2011. doi:10.1155/2011/585919.
3. WHO [Internet]. March 2014 supplement to the 2013 consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection: recommendations for a public health approach [cited 2016 Oct 1]. Available from: http://www.who.int/hiv/pub/guidelines/arv2013/arvs2013upplement_march2014/en/
4. Xu S, Song A, Nie J, Li X, Meng S, Zhang C, et al. Comparison between the automated Roche Cobas AmpliPrep/Cobas TaqMan HIV-1 test version 2.0 assay and its version 1 and Nuclisens HIV-1 EasyQ version 2.0 assays when measuring diverse HIV-1 genotypes in China. J Clin Virol. 2012;53:33-7.
5. Sollis KA, Smit PW, Fiscus S, Ford N, Vitoria M, Essajee S, et al. Systematic review of the performance of HIV viral load technologies on plasma samples. PLoS One [Internet]. 2014 Feb [cited 2016 Oct 1];9(2):[about 9 p]. Available from: http://www.plosone.org/article/fetchObject.action?uri=info:doi/10.1371/journal.pone.0085869&representation=PDF
6. UNAIDS [Internet]. The GAP Report [cited 2016 Oct 1]. Available from: http://www.unaids.org/sites/default/files/en/media/unaids/contentassets/documents/unaidspublication/2014/UNAIDS_Gap_report_en.pdf
7. Roselinda, Jekti RP. Genotype and transmission Human Immunodeficiency Virus-1 in seven provinces in Indonesia. Health Science Indones [Internet]. 2012 Dec [cited 2016 Oct 1];3(2):[about 5 p]. Available from: http://ejournal.litbang.depkes.go.id/index.php/HSJI/article/view/3070/3039
8. Ismail YS, Soetjipto, Wasito EB, Nasronudin. HIV Genotype Analysis from HIV Infected Patients in East Java Area. Jurnal Natural [Internet]. 2012 [cited 2016 Oct 1];12(23):[about 7 p]. Available from: http://jurnal.unsyiah.ac.id/natural/article/view/833/771
9. Cha YJ, Yoo SJ, Sohn YH, Kim HS. Performance evaluation of ExiStation HBV diagnostic system for hepatitis B virus DNA quantitation. J Virol Methods. 2013;193:492-7.
10. Wall GR, Perinpanathan D, Clark DA. Comparison of the QIAGEN artus HIV-1 QS-RGQ test with the Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 test v2.0. J Clin Virol. 2012;55:62-6.
11. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet. 1986;1:307-10.
12. Panel on Antiretroviral Guidelines for Adults and Adolescents [Internet]. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents [cited 2016 Oct 1]. Available from: http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf
13. British HIV Association. British HIV Association guidelines for the treatment of HIV-1-positive adults with antiretroviral therapy. HIV Med. 2014;15(Suppl 1):1-85.
14. Thompson MA, Aberg JA, Hoy JF, Telenti A, Benson C, Cahn P, et al. Antiretroviral treatment of adult HIV infection: 2012 recommendations of the International Antiviral Society-USA Panel. JAMA. 2012;308:387-402.
15. European AIDS Clinical Society [Internet]. Guidelines, version 7.1, November 2014 [cited 2016 Oct 1]. Available from: http://www.eacsociety.org/guidelines/eacs-guidelines/eacs-guidelines.html
16. Amendola A, Marsella P, Bloisi M, Forbici F, Angeletti C, Capobianchi MR. Ability of two commercially available assays (Abbott RealTime HIV-1 and Cobas AmpliPrep/Cobas TaqMan HIV-1 version 2.0) to quantify low HIV-1 RNA levels (<1,000 copies/milliliter): Comparison with clinical samples and NIBSC working reagent for nucleic acid. J Clin Microbiol. 2014;52:2019-26.
17. Ananworanich J, Pancharoen C, Sirivichayakul S, Buranapraditkun S, Apateerapong W, Ubolyam S, et al. Undetectable plasma HIV-1 RNA with strong gag-pol specific interferon-gamma ELISPOT response in an HIV-1 clade A/E-infected child untreated with antiretroviral therapy. Asian Pac J Allergy Immunol. 2004;22:165-9.
18. Laprise C, De Pokomandy A, Baril JG, Dufresne S, Trottier H. Virologic failure following persistent low-level viremia in a cohort of HIV-positive patients: Results from 12 years of observation. Clin Infect Dis. 2013;57:1489-96.
19. Aleman S, Söderbärg K, Visco-Comandini U, Sitbon G, Sönnerborg A. Drug resistance at low viraemia in HIV-1-infected patients with antiretroviral combination therapy. AIDS. 2002;16:1039-44.
20. Delaugerre C, Gallien S, Flandre P, Mathez D, Amarsy R, Ferret S, et al. Impact of low-level-viremia on HIV-1 drug-resistance evolution among antiretroviral treated-patients. PLoS One [Internet]. 2012 [cited 2016 Oct 1];7:[about 6 p]. Available from: http://www.plosone.org/article/fetchObject.action?uri=info:doi/10.1371/journal.pone.0036673&representation=PDF
21. Karlsson AC, Younger SR, Martin JN, Grossman Z, Sinclair E, Hunt PW. Immunologic and virologic evolution during periods of intermittent and persistent low-level viremia. AIDS. 2004;18:981-9.
22. Taiwo B, Gallien S, Aga E, Ribaudo H, Haubrich R, Kuritzkes DR. Antiretroviral drug resistance in HIV-1-infected patients experiencing persistent low-level viremia during first-line therapy. J Infect Dis. 2011;204:515-20.

ORIGINAL ARTICLE

Asian Pacific Journal of
Allergy and Immunology

APJAI

Deciphering critical amino acid residues to modify and enhance the binding affinity of ankyrin scaffold specific to capsid protein of human immunodeficiency virus type 1

Somphot Saoin,[1,2] Tanchanok Wisitponchai,[1,2] Kannaporn Intachai,[1,2] Koollawat Chupradit,[1,2] Sutpirat Moonmuang,[1,2] Sawitree Nangola,[3] Kuntida Kitidee,[4] Kanda Fanhchaksai,[1,2] Vannajan Sanghiran Lee,[5,6] Saw-See Hong,[7,8] Pierre Boulanger,[7,8] Phimonphan Chuankhayan,[9] Chun-Jung Chen,[9,10,11] Chatchai Tayapiwatana[1,2]

Abstract

Background: Ank^{GAG}1D4 is an artificial ankyrin repeat protein which recognizes the capsid protein (CA) of the human immunodeficiency virus type 1 (HIV-1) and exhibits the intracellular antiviral activity on the viral assembly process. Improving the binding affinity of Ank^{GAG}1D4 would potentially enhance the Ank^{GAG}1D4-mediated antiviral activity.

Objective: To augment the affinity of Ank^{GAG}1D4 scaffold towards its CA target, through computational predictions and experimental designs.

Method: Three dimensional structure of the binary complex formed by Ank^{GAG}1D4 docked to the CA was used as a model for van der Waals (vdW) binding energy calculation. The results generated a simple guideline to select the amino acids for modifications. Following the predictions, modified Ank^{GAG}1D4 proteins were produced and further evaluated for their CA-binding activity, using ELISA-modified method and bio-layer interferometry (BLI).

Results: Tyrosine at position 56 (Y56) in Ank^{GAG}1D4 was experimentally identified as the most critical residue for CA binding. Rational substitutions of this residue diminished the binding affinity. However, vdW calculation preconized to substitute serine for tyrosine at position 45. Remarkably, the affinity for the viral CA was significantly enhanced in Ank^{GAG}1D4-S45Y mutant, with no alteration of the target specificity.

Conclusions: The S-to-Y mutation at position 45, based on the prediction of interacting amino acids and on vdW binding energy calculation, resulted in a significant enhancement of the affinity of Ank^{GAG}1D4 ankyrin for its CA target. Ank^{GAG}1D4-S45Y mutant represented the starting point for further construction of variants with even higher affinity towards the viral CA, and higher therapeutic potential in the future.

Keywords: ankyrins; Ank^{GAG}1D4; HIV-1 assembly; capsid; computer-aided molecular design

From:
1 Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand
2 Center of Biomolecular Therapy and Diagnostic, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand
3 Division of Clinical Immunology and Transfusion Sciences, School of Allied Health Sciences, University of Phayao, Phayao, Thailand
4 Center for Research and Innovation, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand
5 Thailand Center of Excellence in Physics, Commission on Higher Education, Bangkok, Thailand
6 Department of Chemistry, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia
7 Viral Infections and Comparative Pathology, UMR-754 UCBL-INRA-EPHE, Université Lyon 1, Lyon Cedex 07, France
8 Institut National de la Santé et de la Recherche Médicale, Paris, France
9 Life Science Group, Scientific Research Division, National Synchrotron Radiation Research Center, Hsinchu, Taiwan
10 Department of Physics, National Tsing Hua University, Hsinchu, Taiwan
11 Institute of Biotechnology and Center for Bioscience and Biotechnology, National Cheng Kung University, Tainan, Taiwan

Corresponding author:
Chatchai Tayapiwatana
Division of Clinical Immunology,
Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand 50200
E-mail: asimi002@hotmail.com

Introduction

Antibodies have been implemented for many medical applications and represented the largest class of therapeutic proteins in clinical development.[1,2] However, therapeutic antibodies have several limitations, such as their relatively high production cost, inefficient penetration and insufficient retention in targeted tissues.[3,4] In addition, the major inconvenience is the requirement of disulfide bond formation to stabilize their structure and biological activity, a biochemical process which cannot occur in the reducing intracellular milieu. To address these limitations, non-immunoglobulin scaffolds have been proposed as alternatives to antibodies.[5,6] These protein scaffolds were selected according to the following criteria: their high degree of solubility and stability, independence of disulfide bonds and/or glycosylation sites, their low cost of production, and their high affinity for their targets.[5]

Ankyrins, and their artificial derivatives designed ankyrin repeat proteins (DARPins), are a novel class of protein binders designed for the recognition of a variety of molecular targets with high specificity and affinity. They are biologically active in various environments, including the extracellular milieu and intracellular compartments.[7-10] Applications of artificial ankyrins to antiviral treatments are a new challenge for the future therapeutics, and recently, antiretroviral ankyrins have been designed to inhibit HIV-1 replication. This is the case for a CD4-specific DARPin, which competes with the HIV-1 envelope gp120 for CD4-receptor binding, and blocks the cell entry of divergent strains of HIV-1.[11] Likewise, a gp120 -reactive DARPin interacting with the V3 loop of gp120 acts as an efficient HIV-1 entry inhibitor.[12]

Ank^{GAG}1D4 is a trimodular repeat protein which was selected from a phage-displayed ankyrin library for its binding to the HIV-1 capsid (CA).[13] Ank^{GAG}1D4 has been shown to possess a significant antiviral effect against HIV-1 replication.[13,14] HIV-1-infected SupT1 cells stably expressing Ank^{GAG}1D4 showed a significant reduction of the viral progeny yield, compared to control SupT1 cells.[13] Likewise, the N-myristoylated version of Ank^{GAG}1D4 was found to negatively interfere with HIV-1 replication in primary human CD4+ T-cells.[14] Ank^{GAG}1D4 showed a relatively high performance in terms of inhibition of viral particle assembly, but its efficacy, in terms of protection from HIV-1 infection, was not maximal, especially at late phase postinfection.

Increasing the affinity of Ank^{GAG}1D4 towards its viral target seemed therefore a well designated strategy to enhance the Ank^{GAG}1D4-mediated antiviral activity to obtain a complete HIV-1 inhibition. Several strategies are available to achieve this goal. In the present study, we used a relatively simple but rational approach through computational design, as an alternative to the time-consuming method of combinatorial design or directed evolution of ankyrin genes, using error-prone PCR in combination with *in vitro* selection of modified ankyrins by ribosome-display or phage-display.[9,11]

The goal of this study was to enhance binding affinity of Ank^{GAG}1D4 towards its viral target, through manipulation of selected amino acids belonging to the binding site or located in its close vicinity. Our choice of amino acid residues to modify in Ank^{GAG}1D4 was guided by the position and nature of the major determinants of the CA binding, deduced from crystal structure and computational analysis. Calculation of the van der Waals (vdW) binding forces guided us in our choice of the amino acid positions to target in the ankyrin sequence. The biological effects of these mutations on the affinity of Ank^{GAG}1D4 towards the CA were evaluated using ELISA-derived immunological tests and bio-layer interferometry. This computationally guided molecular design, which involved molecular structures and vdW binding energy of interacting molecules, provided a rational method for the modification of binding parameters, and represented a gain in time compared to other strategies.

Methods

Structure-guided enhancement of the affinity of Ank^{GAG}1D4 towards the viral CA

Ank^{GAG}1D4 residues, S45, Y56, R89, K122 and K123 were identified as key residues in the CA-binding. All key residues were substituted to the 20 natural amino acids (with the exception of S, Y, R or K, respectively for each case). Y56 was the major key residue of the CA-binding, while the other residues were secondary players in this interaction. These residues were categorized by the frequency of the interaction pairs as described in our previous paper.[15] In the CA/Ank^{GAG}1D4 complexes, the CA structure was fixed, and the Ank^{GAG}1D4 mutants were minimized by the algorithms of Steepest Descent and followed by Conjugate Gradient, based on CHARMM19 force field. The vdW values at positions 45, 56, 89, 122 and 123 in Ank^{GAG}1D4-CA mutants interacting with CA in our simulated models were compared to the residues found at the corresponding positions in parental Ank^{GAG}1D4.

Site-directed mutagenesis of Ank^{GAG}1D4

Plasmid pQE30 encoding the full-length Ank^{GAG}1D4 was used for the amplification of the first ankyrin module (module#1) which contained the S45 or Y56 residues in standard PCR method. PCR was carried out using KOD Hot Start DNA polymerase (Novagen, Madison, WI). After purification, the amplified fragment was ligated into the pTZ57R/T acceptor vector, using the InsTAclone PCR Cloning Kit (Thermo Scientific, Rockford, IL). The ligation product was then introduced into the *E.coli* XL1-blue strain. The correctness of the clones was verified by standard sequencing method. The pTZ57R/T plasmid carrying the Ank^{GAG}1D4 module#1 was purified, and used as template for site-directed mutagenesis of S45 or Y56 residues by using the Quick change® lightening site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Construction of expression vector encoding Ank^{GAG}1D4 mutants

The DNA fragments encoding module#1 mutants were amplified from the corresponding pTZ57R/T plasmids as above. The DNA fragment encoding the second module linked to the third module of Ank^{GAG}1D4 (module#2+module#3) were amplified from the pQE30-Ank^{GAG}1D4 plasmid. The DNA fragments encoding the module#1 mutants and the DNA fragment encoding module#2+module#3 were then assembled using the Gibson Assembly® Master Mix (New England Biolabs, Ipswich, MA). Next, the trimodular sequences carrying the mutations in module#1 were amplified using the KOD Hot Start DNA polymerase (Novagen, Madison, WI) subsequently

treated with *Not*I and *Hind*III. The amplified fragments were ligated with the pQE30 plasmid which had been cleaved with the same enzymes. The ligated products were used to transform XL1-blue competent cells. The new constructs of pQE30-Ank^{GAG}1D4 mutants were transferred to *E. coli* strain M15[pREP4] for recombinant protein expression. The recombinant Ank^{GAG}1D4 mutant proteins were produced and purified as described in detail elsewhere.[13,15]

Production of recombinant CA protein from bacterial cells

The recombinant CA protein was expressed following the protocol previously described.[15] The protein was purified by affinity chromatography on HisTrap column, using ÄKTA Prime™ plus (GE Healthcare, Piscataway, NJ). Protein concentration was measured using the Bradford protein assay kit (Pierce/Thermo Scientific, Rockford, IL) and analyzed by western blotting. In addition, the biotinylated CA was prepared by using EZ-Link™ Sulfo-NHS-LC-Biotinylation kit (Pierce/Thermo Scientific, Rockford, IL) according to the procedure recommended by the manufacturer.

Evaluation of the CA-binding activity of Ank^{GAG}1D4 and Ank^{GAG}1D4 mutants

The binding of Ank^{GAG}1D4 and Ank^{GAG}1D4 mutants to the CA protein was evaluated using AMELIA, a modification of the conventional ELISA, consisting of ankyrin-mediated capture of the target protein, followed by enzyme-linked immunoassay.[15] The microtiter plate was directly coated with recombinant Ank^{GAG}1D4 or Ank^{GAG}1D4 mutants at a final concentration of 5 µg/mL overnight at 4°C. Purified CA protein, diluted in PBS-2% bovine serum albumin (BSA), was added, and incubated at RT for 1h. After incubation, the excess amount of CA was removed using a conventional washing buffer (0.05% Tween-20 in PBS) or a high stringency washing buffer (1% Triton X100 (v/v) and 550 mM NaCl prepared in PBS (136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4), pH7.4).[16] The level of Ank^{GAG}1D4-mediated capture of the target CA protein was detected using the mouse monoclonal antibody against CA protein (M88), followed by HRP-conjugated goat anti-mouse immunoglobulin.

Binding kinetic analysis by bio-layer interferometry (BLI)

The binding kinetics of the Ank^{GAG}1D4 mutants with recombinant CA were measured using BLI performed with the BLItz™ system (ForteBio, Menlo Park, CA). All interaction analyses were conducted in sample diluent (bovine serum albumin (w/v, 2%) and tween-20 detergent (v/v, 0.05%) in PBS). Streptavidin (SA) biosensors were pre-wetted for 15 min in buffer immediately before use. The microplates used in the ForteBio system were filled with sample diluent for 250 µl/well. Biotinylated CA protein at 100 µg/mL was immobilized to Streptavidin (SA) biosensors for 2 min. Next, saturated biosensors were washed in sample diluent for 30 sec and transferred to the tubes containing 10 µg/mL Ank^{GAG}1D4 parental or mutants in diluent buffer. The association and dissociation values of Ank^{GAG}1D4 parental and mutants were measured for each step of 1.30 min. Kinetic parameters (k_{on} and k_{off}) and the equilibrium dissociation constant (K_D) were calculated from a non-linear local fit of the data between Ank^{GAG}1D4 and CA proteins, using the BLItz Pro 1.1 software. To test the specificity, biotinylated interferon-γ (IFN-γ) which was also immobilized to SA biosensor, was used as irrelevant protein target served as negative control.

Determination of the CA ankyrinotope binding of Ank^{GAG}-1D4-S45Y

AMELIA was applied to competitive system for analyzing the binding site on CA of Ank^{GAG}1D4-S45Y mutant. Each of ankyrin including parental Ank^{GAG}1D4, Ank^{GAG}1D4-S45Y and Ank^{GAG}1D4-Y56A (final concentration of 10 µg/mL) was coated overnight at 4°C. Biotinylated CA protein (final concentration of 2.8 µg/mL) was added and incubated at RT for 1 h. After washing with high stringency buffer, the competitors in solution comprising recombinant Ank^{GAG}1D4, Ank^{GAG}1D4-S45Y, Ank^{GAG}1D4-Y56A and interferon-γ (IFN-γ), irrelevant protein (R&D Systems, Minneapolis, MN), at final concentration of 10 µg/mL were individually added to each well. The wells were washed and followed by addition the HRP-conjugated anti-biotin.

Results

Choice of amino acid residue(s) as target(s) for editing

Tyrosine at position 56 (Y56) in Ank^{GAG}1D4 was identified by molecular docking analysis as the key residue contributing the most to the binding of Ank^{GAG}1D4 to its viral partner.[15] The first step of our study was to experimentally verify that Y56 really played a crucial role in this interaction. If confirmed, this would designate Y56 as a candidate residue for the modulation of the binding affinity of Ank^{GAG}1D4 towards the CA. To this aim, we generated the substitution mutant Ank^{GAG}1D4-Y56A by site-directed mutagenesis of tyrosine at position 56 into alanine. The binding reactivity of Ank^{GAG}1D4-Y56A recombinant protein to CA was evaluated by AMELIA, an ELISA-modified method based on the ankyrin-mediated capture of the target protein, followed by enzyme-linked immunoassay.[15] The parental ankyrin Ank^{GAG}1D4 showed a high CA-binding activity towards the viral CA protein, and the interaction observed between the two partners occurred in a CA concentration-dependent manner. In contrast, no CA-binding activity was detectable for the Ank^{GAG}1D4-Y56A mutant, at all CA concentrations tested (**Figure 1A**). This confirmed experimentally our previous prediction that Y56 was a first-rank key residue for the binding of HIV-1 CA,[15] and designated this residue as the best candidate for modification.

To further investigate on the role of residue Y56 in the binding of Ank^{GAG}1D4 to the CA, Ank^{GAG}1D4-Y56A mutant protein was purified to homogeneity and crystallized, and a structural analysis Ank^{GAG}1D4-Y56A crystal was performed. The structure of Ank^{GAG}1D4-Y56A crystal was determined at 1.9 Å resolution (accession codes 4ZFH). All X-ray crystallographic data and refinement statistics are summarized in **Table 1**. The result demonstrated that the overall structure of Ank^{GAG}1D4-Y56A was similar to that of Ank^{GAG}1D4 parental, as shown by the superimposition of the two crystal structures. The root mean square deviation (RMSD) of the Cα backbone between Ank^{GAG}1D4-Y56A mutant and Ank^{GAG}1D4 parental was ~ 0.49 Å (**Figure 1B**), and no significant structural

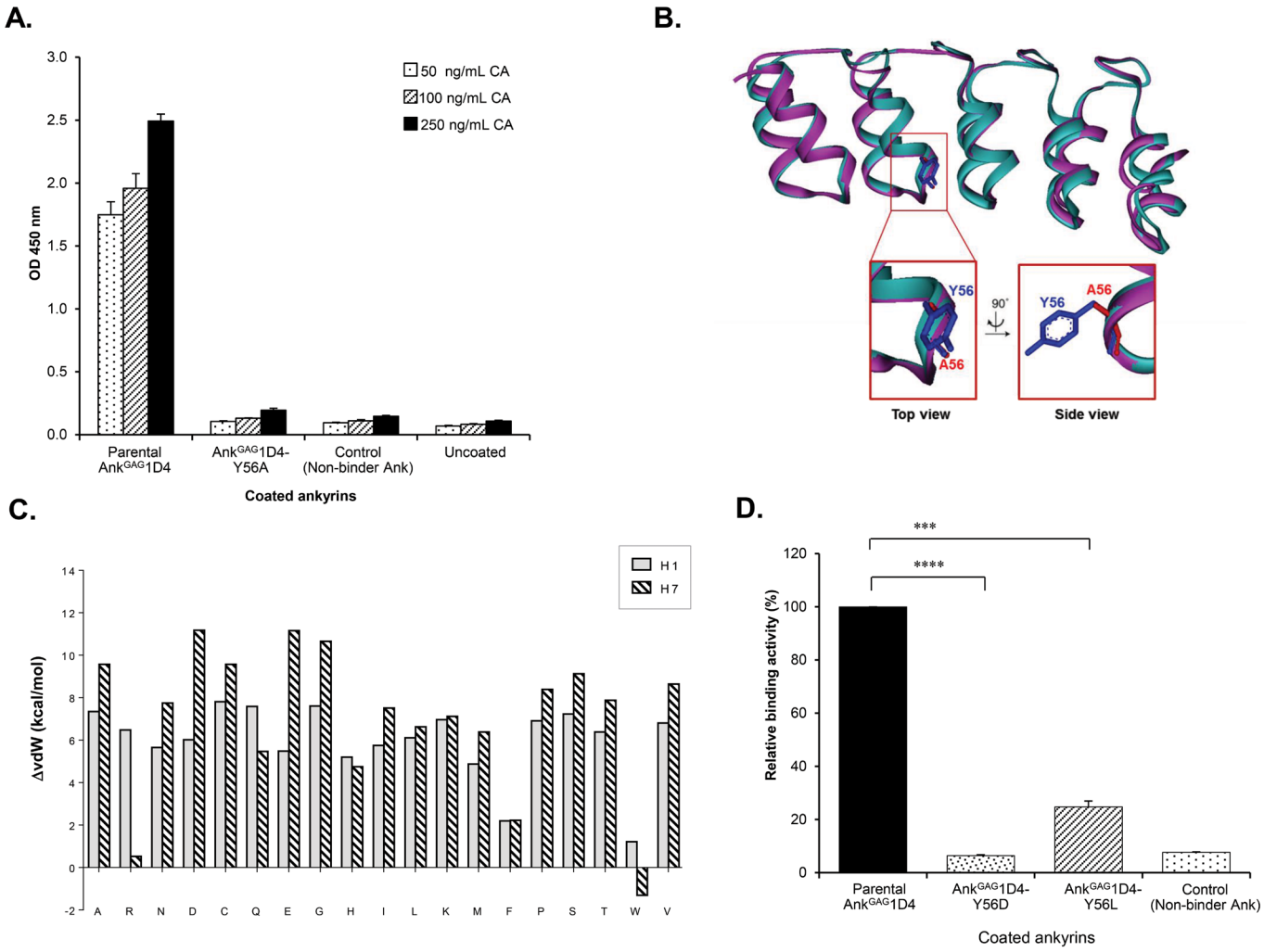


Figure 1. Effect of tyrosine substitution at position 56 on the interaction of Ank^{GAG}1D4 with the CA protein of HIV-1. (A) The CA-binding activity of the Ank^{GAG}1D4-Y56A mutant was compared with that of parental Ank^{GAG}1D4, using AMELIA. Samples of ankyrin proteins, parental Ank^{GAG}1D4, Ank^{GAG}1D4-Y56A mutant, and a non-binder ankyrin as negative control, were used at a concentration of 5 µg/mL, and coated on ELISA plate. After rinsing, CA protein was added to each well at increasing concentrations. Ankyrin-bound CA was detected using anti-CA mouse monoclonal antibody, followed by HRP-conjugated goat anti-mouse immunoglobulin. Negative control consisted of an irrelevant ankyrin protein which did not bind to CA. Results presented are from triplicate experiments (mean ± SD). (B) Superimposition of the crystal structures of parental Ank^{GAG}1D4 (cyan) and Ank^{GAG}1D4-Y56A mutant (purple), in ribbon-style representation. The square boxes show close-up views of Y56 (blue) and A56 (red) residues in the superimposed crystal structures. (C) Screening of amino acid substituents at position 56 in the Ank^{GAG}1D4 binding surface and assessment of their CA-binding activity. The differences in the vdW energy between mutant residues and Y56 were calculated for the H1-mediated (filled bars) and H7-mediated (hatched bar) modes of CA-binding, using the CHARMM19 force field program and the Momany-Rone algorithm. (D) CA-binding activity of selected Ank^{GAG}1D4 substitution mutants at position 56, analyzed by AMELIA. The binding activity was measured at OD_{450}, and the signal obtained with parental Ank^{GAG}1D4 and CA protein was attributed the 100% value. The relative binding activity was calculated using the following formula: $[OD_{mutant}/OD_{parental}]\times100$. Data presented are from triplicate experiments (mean ± SD), analyzed using one-way ANOVA. ***$P < 0.001$ and ****$P < 0.0001$.

alteration resulted from the substitution of tyrosine by alanine. This strongly suggested that the loss of CA-binding activity in Ank^{GAG}1D4-Y56A mutant was due to the loss of the tyrosine ring at position 56, and not to the overall conformation of the ankyrin mutant molecule.

Substitutions of the first-rank key residue at position 56: predictions and experimental results

The computational prediction using 3D docking structures and calculation of vdW binding forces suggested that none of the 19 natural amino acids which would substitute for Y56 would confer any significant affinity improvement to the Ank^{GAG}1D4 mutants (**Figure 1C**). This was experimentally confirmed by introducing some representatives of these substitutions into the recombinant ankyrin protein, viz. Y56D and Y56L. These two ankyrin mutants had lost their capacity to bind to the CA (**Figure 1D**). The results demonstrated that Y56 was the most critical residue in the interaction with CA partner and could not be substituted for any other amino acid without profoundly altering the affinity.

Table 1. Statistics of X-ray diffraction data and structurerefinement of Ank^{GAG}1D4-Y56A mutant.[(a)]

Data collection	
Space group	C2
Cell dimension (Å)	a = 104.84, b = 28.64, c = 53.54 β = 106.39°
Resolution range (Å)	50–1.89
Observed reflections Total Unique	 58,430 11,686
Completeness (%)	98.2 (96.2)
Redundancy	5.0 (4.8)
R_{sym} (%)	5.6 (22.9)

Refinement	
Resolution range (Å)	50–1.89
R_{work}/R_{free} (%)	21.1/27.6
Average B-factor (Å^2)	20.9
RMS deviation from ideal geometry Bond lengths (Å) Bond angle (°)	 0.016 1.87

(a) Values in parenthesis in the right column correspond to the highest-resolution shell (1.94–1.89 Å).

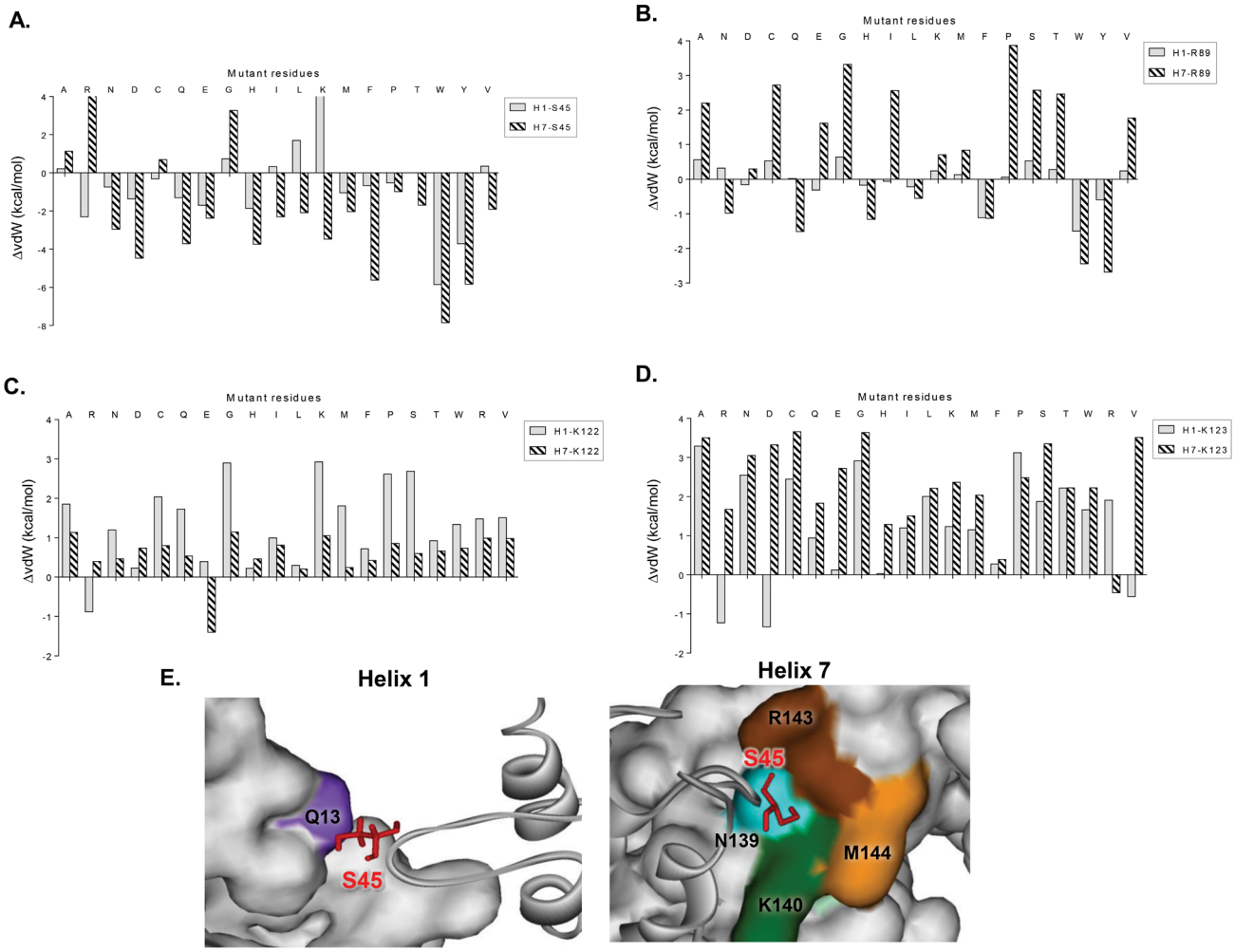


Figure 2. Screening of amino acid substituents at position 45, 89, 122 and 123 in Ank^{GAG}1D4. (A-D) Bar-graph of the ΔvdW values between mutant residues and serine 45 (A), arginine 89 (B), lysine 122 (C) and lysine 123 (D). The binding energy optimization was performed by screening 19 natural amino acid residues at these four positions in Ank^{GAG}1D4, when bound to the CA protein *via* the H1 or H7 alpha-helix of the CA N-terminal domain. The differences in the vdW energy between each one of the 19 mutant residues and the indicated parental residue were calculated for the H1-mediated (filled bars) and H7-mediated (hatched bars) modes of CA-binding, using the CHARMM19 force field program and the Momany-Rone algorithm. (E) Pocket analysis within 5 Å-distance of serine at position 45 (S45) in Ank^{GAG}1D4 binding to CA target. Red (in stick style representation) on ribbon style of Ank^{GAG}1D4 belongs to S45 residues. The amino acids surrounding S45 residue on CA protein representing in surface style are shown by different colors in H1-mediated (left panel) and H7-mediated (right panel) modes of CA-binding.

Substitutions of second-rank key residues

The results obtained with mutants at position 56 led us to consider substitutions of residues S45, R89, K122 and K123, which were identified as second-rank determinants of the interaction of Ank^{GAG}1D4 with CA, compared to Y56.[15] As for the amino acid screening at position 56, these four residues were substituted by a panel of 19 natural amino acids, and the corresponding vdW energy calculated and compared to that of the parental Ank^{GAG}1D4. Mutant residues with ΔvdW values lower than -2 kcal/mol were considered as good candidates for conferring to Ank^{GAG}1D4 mutants a higher affinity to the CA (**Figure 2A-2D**). As previously, the selected mutations were introduced in the Ank^{GAG}1D4 gene, and the recombinant protein mutants analyzed in vitro for CA binding activity. In the case of K122 and K123, there was no substituent providing a ΔvdW lower than -2 kcal/mol (**Figure 2C and 2D**). Likewise, R89 substitution by tryptophan or tyrosine residue resulted in ΔvdW values of -2.68 and -2.43 kcal/mol, respectively. These two values were only slightly lower than the threshold value of -2 kcal/mol, and were only obtained in the H7-mediated mode of CA-binding (**Figure 2B**).

In the case of S45 however, 11 amino acid substituents out of 19 showed a ΔvdW value significantly lower than -2 kcal/mol (**Figure 2A**). This implied that position 45 represented a privileged target for the modulation of the binding affinity of Ank^{GAG}1D4 towards the CA. In both H1- and H7-mediated modes of CA binding, tryptophan and tyrosine residues were the two residues at position 45 which showed the lowest ΔvdW values: -5.85 and -3.72 kcal/mol, respectively, in the H1-mediated mode of CA binding; -7.85 and -5.84 kcal/mol, respectively, in the H7-mediated mode (**Figure 2A**).

Position 45 corresponded to an interfacial residue located on an external loop of the Ank^{GAG}1D4, and of ankyrin molecules in general. Sequence comparison showed that the residues at position 45 in 96% of 500 ankyrin sequences available in Uniref90 database analyzed by ConSurf server[17,18] were more hydrophilic than hydrophobic[19] (data not shown). Tyrosine is also less hydrophobic than tryptophan, therefore S45Y was selected as mutant, and the recombinant protein mutant Ank^{GAG}1D4-S45Y was generated as described above. As a further support to the vdW calculation-based prediction of S45 mutants, pocket analysis at position 45 showed that protein interaction within the 5Å-distance with ankyrin involved Q13 residue in the H1-mediated CA-ankyrin complex, and N139, K140, R143, M144 residues in the H7-mediated CA-ankyrin complex. Both pockets contributed to the binding through vdW bonds rather than through electrostatic interactions (**Figure 2E**).

Evaluation the effect of S-to-Y replacement at position 45 of Ank^{GAG}1D4 mutant on the CA-binding activity

To verify these predictions, the binding affinity of the Ank^{GAG}1D4-S45Y mutant towards the CA was evaluated by AMELIA in which a high-stringency washing buffer was introduced in the washing step, in order to eliminate non-specific binding, and to discriminate between high and low affinity binder.[16] The results showed that the binding activity of Ank^{GAG}1D4-S45Y was significantly higher than parental Ank^{GAG}1D4, 60% under conventional conditions and 110 % under high stringency conditions (**Figure 3**).

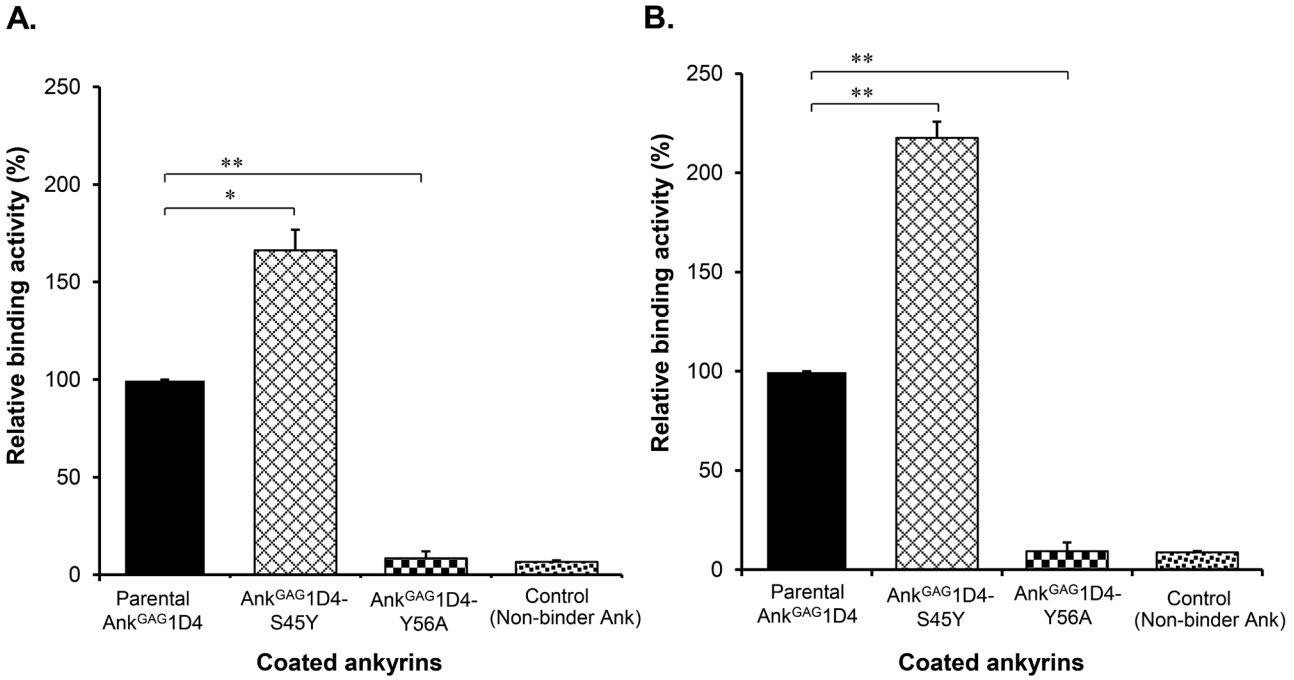
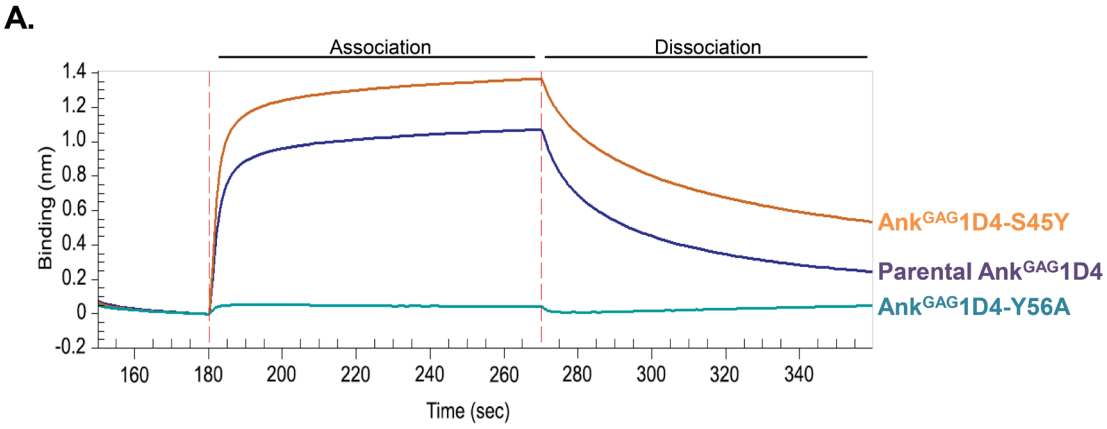


Figure 3. Effect of S-to-Y substitution at position 45 on the CA-binding activity of Ank^{GAG}1D4-S45Y mutant. The binding activity of Ank^{GAG}1D4 substitution mutants at position 45 was assessed by AMELIA using a conventional washing buffer (A), or a high stringency washing buffer (B). The binding activity was measured at OD_{450}, and the signal obtained with parental Ank^{GAG}1D4 and CA protein was attributed the 100% value. The relative binding activity was calculated using the following formula: [OD_{mutant}/$OD_{parental}$]×100. Results presented are from triplicate experiments (mean ± SD), analyzed using one-way ANOVA. *P < 0.05, **P < 0.01.

To confirm the augmentation of Ank^{GAG}1D4-S45Y affinity towards the CA, the CA-binding kinetics of the Ank^{GAG}1D4-S45Y mutant was evaluated by bio-layer interferometry using the BLItz™ system. The kinetic analysis showed that the equilibrium dissociation constant of the binding reaction between CA and Ank^{GAG}1D4-S45Y was found to be $K_D \approx 45$ nM. Ank^{GAG}1D4-S45Y mutant therefore exhibited a significantly higher binding affinity towards the CA, compared to parental Ank^{GAG}1D4 (2.4-fold; **Figure 4**). The kinetic parameters could not be determined for the Ank^{GAG}1D4-Y56A mutant due to the absence of detectable interaction corresponding to **Figure 1A**. Of note, Ank^{GAG}1D4 and Ank^{GAG}1D4-S45Y were both unable to bind recombinant interferon-γ (IFN-γ) which was the irrelevant protein served as negative control (data not shown). This result demonstrated that the S-to-Y mutation at position 45 established a new variant of Ank^{GAG}1D4 which had a superior binding activity towards the HIV-1 CA protein with no change the target specificity, compared to parental Ank^{GAG}1D4.

Viral CA binding site recognition by the Ank^{GAG}1D4-S45Y mutant

The S-to-Y mutation at position 45 in Ank^{GAG}1D4-S45Y might affect the recognition of its CA binding site (referred to as ankyrinotope),[15] and result in the binding of this mutant to a region of the CA different from the initial binding site of the parental Ank^{GAG}1D4. The nature of ankyrinotope(s) of parental Ank^{GAG}1D4 and Ank^{GAG}1D4-S45Y mutant was determined by competitive AMELIA. In the case of Ank^{GAG}1D4-coated conditions, the result showed that both Ank^{GAG}1D4-S45Y mutant and parental Ank^{GAG}1D4 used as competitors significantly diminished the binding activity of Ank^{GAG}1D4 towards the CA protein (**Figure 5**). Interestingly, the ability of Ank^{GAG}1D4-S45Y to compete with Ank^{GAG}1D4 for CA binding was significantly higher than that of the homologous competition of Ank^{GAG}1D4 with itself. No decrease in the binding signal was observed in competition assays with IFN-γ used as the negative control. In the case of Ank^{GAG}1D4-S45Y



B.

Variants	$k_{on} \times 10^4$ (1/Ms)	$k_{off} \times 10^{-3}$ (1/s)	K_D (nM)
Parental Ank^{GAG}1D4	30.5 ± 5.1	32.8 ± 1.3	109.2 ± 14.9
Ank^{GAG}1D4 -S45Y	40.7 ± 4.1	18.5 ± 0.6	45.6 ± 3.1
Ank^{GAG}1D4 -Y56A	BDL	BDL	BDL

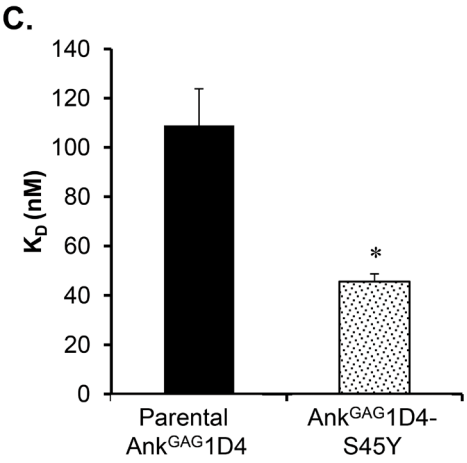


Figure 4. Comparison of CA-binding kinetics of parental Ank^{GAG}1D4 and Ank^{GAG}1D4-S45Y mutant. Binding kinetics were performed using the BLItz™ system. Biotinylated CA protein was immobilized on streptavidin-biosensors and subsequently reacted with recombinant Ank^{GAG}1D4 or Ank^{GAG}1D4-S45Y at 10 μg/mL. (A) Representative sensorgrams displaying the kinetics of the association and dissociation of parental Ank^{GAG}1D4, Ank^{GAG}1D4-S45Y and Ank^{GAG}1D4 Y56A mutants towards biotinylated CA protein. Binding curves were locally fit to a 1:1 binding model and analyzed by the BLItz™ Pro 1.1 software. (B) Kinetic parameters of the binding of Ank^{GAG}1D4-S45Y to the CA protein. BDL indicates that the signals obtained were below detection limit. (C), Comparison the equilibrium dissociation constant values (K_D) of parental Ank^{GAG}1D4 and Ank^{GAG}1D4-S45Y, presented as mean ± SD. Data were analyzed using the Student's t test (*$P < 0.05$).

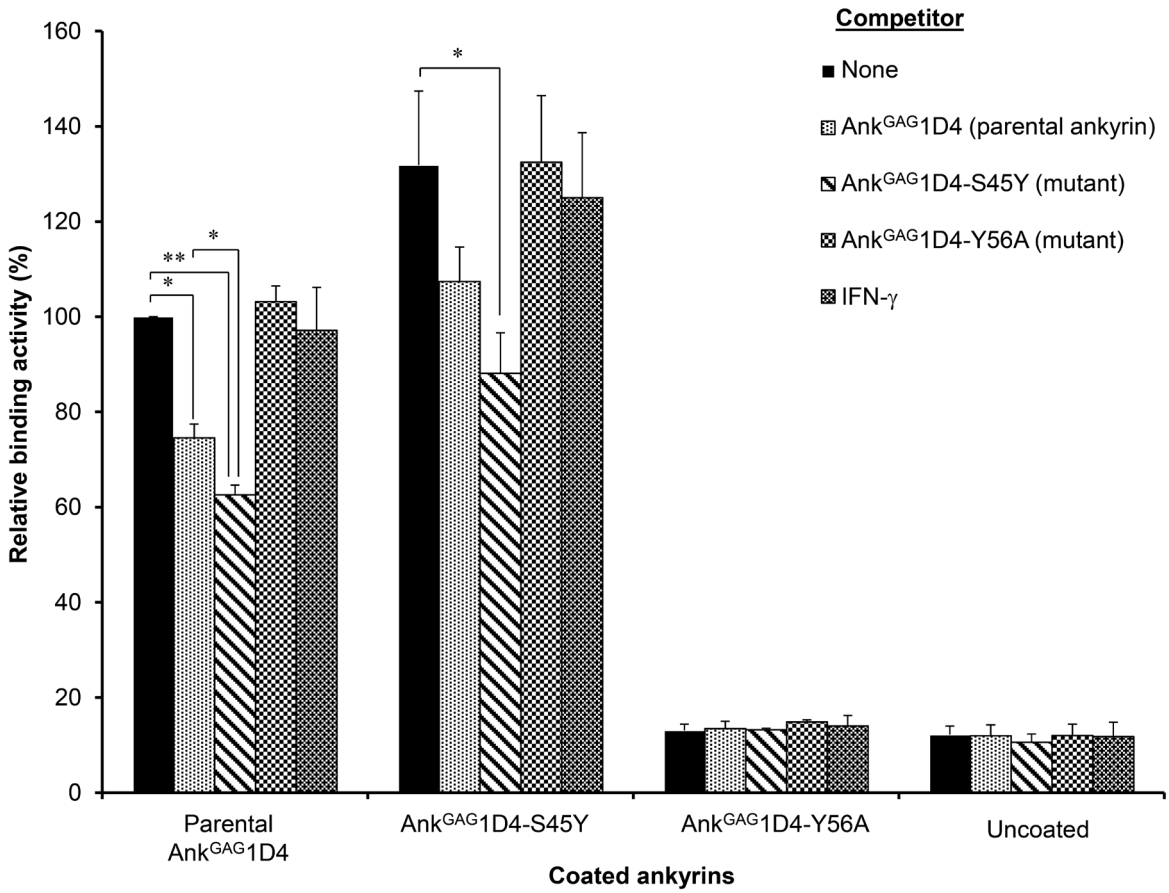


Figure 5. CA ankyrinotope in parental Ank^{GAG}1D4 and Ank^{GAG}1D4-S45Y mutant determined by competitive AMELIA. Immobilized Ank^{GAG}1D4, Ank^{GAG}1D4-S45Y or Ank^{GAG}1D4-Y56A were reacted with biotinylated CA. After removal of the unbound CA, parental or mutant ankyrins were added as competitors for ankyrin-bound CA. Non-binder IFN-γ protein was used as negative control. Ankyrin-bound, biotinylated CA on the solid phase was detected using anti-biotin-HRP. The signal obtained with parental Ank^{GAG}1D4-bound CA in the absence of competitor was attributed the 100% value. The relative binding activity was calculated by the following formula: [$OD_{\text{parental w/ competitors}}$ or $OD_{\text{mutant w/ or w/o competitors}}$/$OD_{\text{parental w/o competitor}}$]×100. All washing steps were performed using high stringency buffer. Results presented are from triplicate experiments (mean ± SD). Data were analyzed using one-way ANOVA. *$P < 0.05$, **$P < 0.01$.

mutant-coated conditions, only the homologous competition of Ank^{GAG}1D4-S45Y with itself significantly decreased the CA binding activity. Taken together, our results indicated (i) that the Ank^{GAG}1D4-S45Y mutant and parental Ank^{GAG}1D4 recognized the same ankyrinotope on the CA protein, and (ii) that Ank^{GAG}1D4-S45Y mutant bound to the CA with a significantly higher affinity compared to parental Ank^{GAG}1D4.

Discussion

The effectiveness of protein scaffolds essentially depends on their affinity for their targets. The affinity of ankyrins can be enhanced by using various methods, including molecular design, to obtain picomolar range values for the affinity constant.[9] Several reports have described successful improvement of the binding affinity of interacting proteins using computationally guided molecular design.[20-24] Computational design is perfectly adapted to this particular goal, since individual amino acid residue(s), or limited protein domain(s), could be targeted and re-designed while maintaining the folding and three-dimensional structure of the whole molecule and its biological properties.[25]

As applied to our antiviral scaffold Ank^{GAG}1D4, a binder of the HIV-1 CA protein,[13,14] the logical start-point of our affinity enhancement strategy was the modification of amino acid residues which were crucial determinants of the interaction of Ank^{GAG}1D4 with the CA protein. These key residues are the main contributors to the binding energy,[20] and were previously identified in the Ank^{GAG}1D4 molecule docked to its CA target.[15] Tyrosine at position 56 (Y56), the first-rank key residue of the binding surface of Ank^{GAG}1D4,[15] was the first and privileged candidate for site-directed mutagenesis. However, the Y-to-A mutation at position 56 totally abolished the binding activity to the CA target. This result suggested that the first-rank key residue(s) responsible for the ankyrin-target stable interaction was untouchable, and that any type of substitution at such key position(s) would be detrimental to the binding reaction of ankyrin with its partner. This might be considered as a general guideline for future ankyrin re-engineering.

As a corollary, modifications of second-rank key residues seemed to be the best option to enhance the binding affinity

of a given ankyrin to its specific target. S45, R89, K122 and K123 belonged to this class of second-rank key residues of the CA-Ank^{GAG}1D4 interaction.[15] For all positions defined as second-rank key residues, computational predictions revealed that S45Y substitution might confer a significant advantage over the parental Ank^{GAG}1D4 in terms of affinity towards the CA. This was experimentally confirmed using the recombinant mutant Ank^{GAG}1D4-S45Y. The equilibrium dissociation constant of the CA-Ank^{GAG}1D4-S45Y binding reaction was found to be in the nanomolar range of values (K_D = 45 nM), i.e. 2.4-fold lower than with parental Ank^{GAG}1D4. Although modest, this augmentation of affinity was significant and would likely lead to net changes in their biological activities.[26-28] Several studies have shown that combined mutations, and not a single one, are necessary to obtain important increases in the affinity between protein partners.[23,29] Mutation(s) of additional key residues in the Ank^{GAG}1D4-S45Y mutant backbone are therefore envisaged to further enhance the affinity of the Ank^{GAG}1D4-S45Y mutant for its HIV-1 target. These mutations should definitely respect the integrity of the site where the first-rank key residue Y56 resides.

The structural model of HIV-1 CA used in our previous and present studies was the monomeric form of crystallized CA_W184/M185A protein (PDB code: 2LF4), consisting of two independently folded domains, the N-terminal (CA^{NTD}) and C-terminal CA (CA^{CTD}) domains, joined by a flexible linker (YSPTS). Due to this linker, the CA monomer can occur under several isoforms. The three CA isoforms that we have previously chosen among the 20 possible different structures for our simulation of CA-Ank^{GAG}1D4 docking complexes provided an adequate representation of all CA-Ank^{GAG}1D4 complex candidates, and our results suggested that the parental Ank^{GAG}1D4 ankyrin was able to interact with any isoform of HIV-1 CA.[15] Our present results suggested that the S-to-Y mutation at position 45 did not alter the specificity of binding of the Ank^{GAG}1D4-S45Y mutant to the CA as the superimposition between Ank^{GAG}1D4-S45Y (PDB ID 5GIK) and parental Ank^{GAG}1D4 (PDB ID 4HLL) showed 0.16 Å, and that both mutant and parental Ank^{GAG}1D4 recognized the same binding site on the CA protein, located in helix 1 and/or 7 of CA^{NTD}, as previously determined.[15]

In conclusion, we delineated the way to enhance the binding affinity of ankyrin scaffolds to their targets *via* computational design. This new strategy integrated *in silico* predictions through manipulation of key residues combined with vdW binding energy calculation, protein engineering technology and biophysical principle based on bio-layer interferometry analysis. This cross-disciplinary approach opened the way to further modifications and improvement of the binding affinity of next generation ankyrin scaffolds for therapeutic uses in the future. With respect to anti-HIV-1 biotherapy, modified antiviral ankyrins could be introduced into T-cells by episomal or lentiviral vectors in order to generate ankyrin-expressing, HIV-1-resistant cells, as described in our previous studies.[13,14] To validate our concept, such antiviral ankyrins will be applied to hematopoietic stem cells for future clinical applications.[30]

Acknowledgements

The work in Thailand was supported by the National Research Council of Thailand (NRCT), the Cluster and Program Management Office (CPMO), the National Science and Technology Development Agency (NSTDA), the Health Systems Research Institute (HSRI), the National Research University project under the Thailand's Office of the Commission on Higher Education, and the Thailand Research Fund through the Royal Golden Jubilee Ph.D. program (grant number PHD/0146/2556 to K.C. and PHD/0184/2557 to S.M.). The work in Malaysia was supported by the Fundamental Research Grant Scheme (FRGS; grant number FP007-2015A). We are grateful to thank Professor Dr. Watchara Kasinrerk for kindly providing monoclonal antibody against HIV-1 capsid protein. The authors would like to thank the Biomedical Technology Research Center, Faculty of Associated Medical Sciences, Chiang Mai University for giving them access to the BLItz™ system, and also thank Loei Hendrick for his valuable advice in the analysis of BLItz™ results.

Conflict of Interests

The authors declare that they have no conflict of interest.

References

1. Nelson AL, Dhimolea E, Reichert JM. Development trends for human monoclonal antibody therapeutics. Nat Rev Drug Discov. 2010;9:767-74.
2. Reichert JM. Antibodies to watch in 2014. MAbs. 2014;6:5-14.
3. Vaughan AT, Cragg MS, Beers SA. Antibody modulation: limiting the efficacy of therapeutic antibodies. Pharmacol Res. 2015;99:269-75.
4. Chames P, Van Regenmortel M, Weiss E, Baty D. Therapeutic antibodies: successes, limitations and hopes for the future. Br J Pharmacol. 2009;157:220-33.
5. Gilbreth RN, Koide S. Structural insights for engineering binding proteins based on non-antibody scaffolds. Curr Opin Struct Biol. 2012;22:413-20.
6. Jost C, Pluckthun A. Engineered proteins with desired specificity: DARPins, other alternative scaffolds and bispecific IgGs. Curr Opin Struct Biol. 2014;27:102-12.
7. Binz HK, Amstutz P, Kohl A, Stumpp MT, Briand C, Forrer P, et al. High -affinity binders selected from designed ankyrin repeat protein libraries. Nat Biotechnol. 2004;22:575-82.
8. Schweizer A, Roschitzki-Voser H, Amstutz P, Briand C, Gulotti-Georgieva M, Prenosil E, et al. Inhibition of caspase-2 by a designed ankyrin repeat protein: specificity, structure, and inhibition mechanism. Structure. 2007;15:625-36.
9. Zahnd C, Wyler E, Schwenk JM, Steiner D, Lawrence MC, McKern NM, et al. A designed ankyrin repeat protein evolved to picomolar affinity to Her2. J Mol Biol. 2007;369:1015-28.
10. Amstutz P, Binz HK, Parizek P, Stumpp MT, Kohl A, Grutter MG, et al. Intracellular kinase inhibitors selected from combinatorial libraries of designed ankyrin repeat proteins. J Biol Chem. 2005;280:24715-22.
11. Schweizer A, Rusert P, Berlinger L, Ruprecht CR, Mann A, Corthesy S, et al. CD4-specific designed ankyrin repeat proteins are novel potent HIV entry inhibitors with unique characteristics. PLoS Pathog. 2008;4:e1000109.
12. Mann A, Friedrich N, Krarup A, Weber J, Stiegeler E, Dreier B, et al. Conformation-dependent recognition of HIV gp120 by designed ankyrin repeat proteins provides access to novel HIV entry inhibitors. J Virol. 2013;87:5868-81.
13. Nangola S, Urvoas A, Valerio-Lepiniec M, Khamaikawin W, Sakkhachornphop S, Hong SS, et al. Antiviral activity of recombinant ankyrin targeted to the capsid domain of HIV-1 Gag polyprotein. Retrovirology. 2012;9:17.
14. Khamaikawin W, Saoin S, Nangola S, Chupradit K, Sakkhachornphop S, Hadpech S, et al. Combined antiviral therapy using designed molecular scaffolds targeting two distinct viral functions, HIV-1 genome integration and capsid assembly. Mol Ther Nucleic Acids. 2015;4:e249.